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13. ABSTRACT ***(Maximum 200 Words)***

The mechanism for genetic errors and genomic instability in breast cancer cells have not been fully delineated. Defects in DNA polymerase δ and its accessory proteins could contribute to the molecular etiology of breast cancer. DNA polymerase δ and its accessory proteins are involved in both DNA replication, repair, recombination and transcription. We are studying this problem in a multifaceted manner at the protein, message and gene level. Both the message and protein of the catalytic subunit of pol δ respond to DNA damage. After damage, the protein level of p53 goes up while that of pol δ goes down. The tumor suppressor Rb also interacts with pol δ. It is hoped that these studies will provide a deeper understanding of the linkage between the regulation of polymerase δ and its accessory proteins and carcinogenesis in breast cancer.

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Marietta Lee 9-20-99

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5. Introduction

The major hypothesis that underlies the proposed studies is that defects in DNA polymerase delta and its accessory proteins could contribute to the molecular etiology of sporadic and hereditary breast tumors. We have been using a polymerase delta immunoaffinity column and immunoprecipitation studies to link DNA replication to the cell cycle. Understanding the cell cycle leads to greater understanding of cancer. Tracking down new cell cycle genes and studying their function in the cell cycle will help to increase our understanding of this basic biological process at both the molecular and genetic levels.

The p53 tumor suppressor is involved in cell cycle arrest, differentiation and apoptosis. It transactivates many genes that regulate cell cycle and cell growth. POLD1 is the gene for the catalytic subunit of pol δ. The 1.8 kb POLD1 gene promoter is TATA-less and CCAAT-less, GC rich and has 32 % promoter activity compared with the SV40 promoter. It contains several putative binding sites for transcriptional factors such as Sp1, Ap1, ATP/CREB. E2F and p53. We have been studying the effect of DNA damaging agents on the message level of p53 and POLD1 and found very interesting results. These results were also true at the protein level. As p53 levels increase, the polymerase δ level decreases. Thus, the repression of the activity of the POLD1 promoter by 53 was studied.

6. **Body**

During the past year we have accomplished the following:

Technical Objective 1 Determining the activity, protein and mRNA levels of pol δ , PCNA, RPA, RFC in normal and breast cancer cell lines and tissue before and after challenge with DNA damaging agents.

Task 1. Assay of DNA polymerase and exonuclease activities , protein and mRNA levels.

This work has been initiated and has been described in our Sept 1998 progress report. We have extended these studies and measured the message level of the p125 catalytic subunit of pol δ after treatment with 30 uM N'-methyl N'nitro-N-nitrosoguanidine (MNNG) and 100 μg/ml methyl methanesulfonate (MMS). Different time points were taken at 0, 0.5 , 1, 2, 3, 4 and 6 hours (Fig. 1). We observed that the message level of MCF 7 declined after treatment with MMS. More interestingly, the protein level of p53 increased after damage treatment. Thus there may be a link between the increase of p53 and the decrease in polymerase δ message.

The levels of polymerase δ protein were also determined by Western blot in MCF 7 cells treated with MMS and MNNG. The Pol δ protein decreased from 0 , 0.5, 1, 2, 3, 4 and 6 hours as the p53 protein level increases after treatment with 100 μg/ml MMS. The same is true after treatment with MNNG (Fig .2) The protein level of Pol δ after MMS and MNNG treatment were also determined in MCF 10A cells and the results were shown in (Fig. 3).

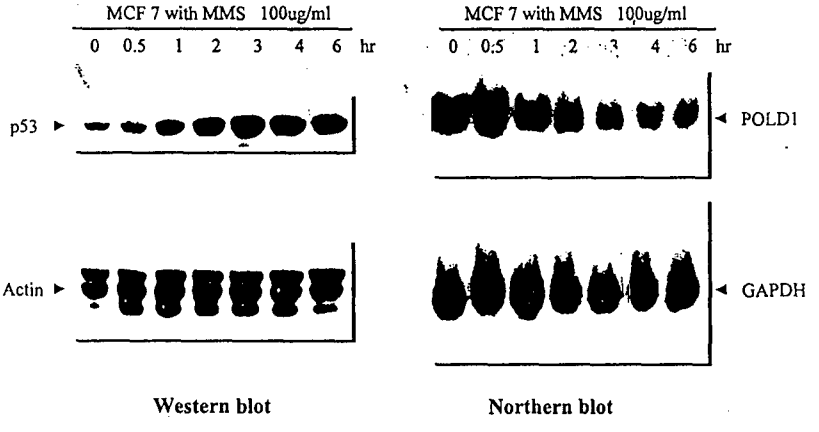

Fig. 1

Fig. 1. Correlation between accumulated p53 and POLD1 mRNA inhibition in cellular response to DNA damaging agents.
Right panel: MCF 7 cells were grown to 90% confluence and treated with 100 μg/ml MMS. At 0, 0.5 1, 2, 3, 4 and 6 hrs the cells were harvested. RNA was then extracted from the cells using RNA-STAT60. 20 μg of RNA was subjected to Northern blotting using POLD1 probe (upper Panel) GAPDH was also used as probe for control (lower panel).
Left Panel: 30 μg of each sample lysate was loaded onto a 10 % SDS-PAGE gel. The protein level of p53 was determined using Western blot with p53 DO-1 monoclonal antibody (upper panel) . Actin antibody was also used as a control on the same blot (lower panel).

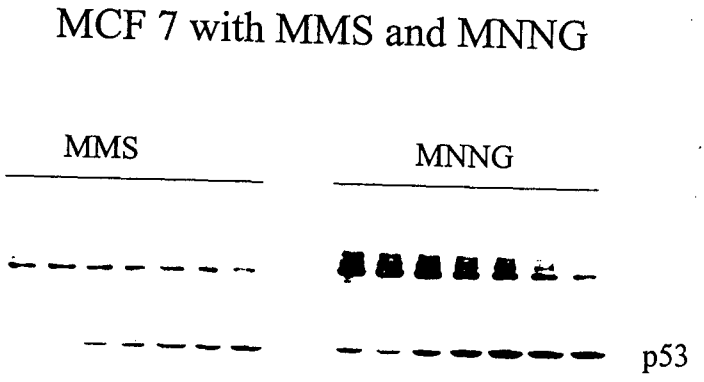

Fig. 2

Fig. 2. Inverse correlation between p53 protein levels and pol δ p125 protein level in response to DNA damaging agents. MCF 7 cells were grown to 90% confluence and treated with 100 μg/ml MMS right panel and 30 μM MNNG. At 0, 0.5 1, 2, 3, 4 and 6 hrs the cells were harvested . Protein was extracted and dissolved in SDS sample buffer. SDS-PAGE was performed. The upper panels were Western blotted with Pol δ p125 antibody and the lower panels were western blotted with p53 antibody.

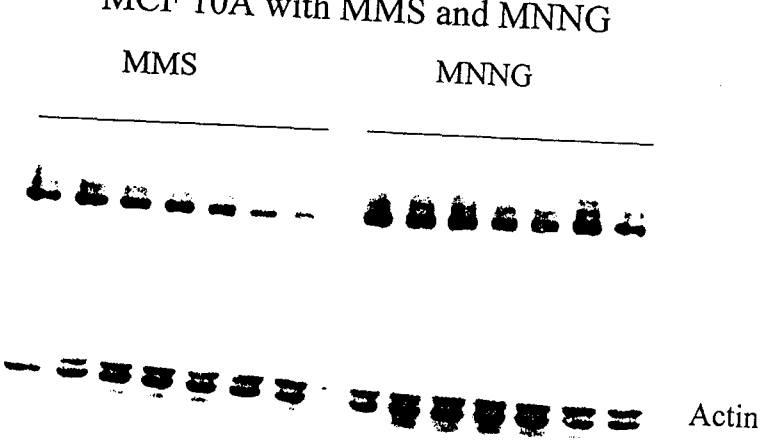

Fig. 3

Fig. 3. Correlation between p53 protein level and Pol δ p125 protein level in response to DNA damaging agents. Same as Fig 2 except MCF 10 A cells were used.

Task 2. Comparison of DNA replication functions of purified pol δ and PCNA of normal and breast cancer cells.

We are in the process of developing methods to measure pol δ enzyme activity after immunoprecipitation. We had developed an immunoaffinity column to purify DNA polymerase δ (Mo et al., submitted). We had used this column to isolate pol δ holoenzyme and multiprotein complexes. However, this procedure is only suitable for a large amount of cells or tissue. We are trying to scale this down so that we can compare the DNA replication functions of normal and breast cancer cells with the least amount of material possible. This is just a matter of technique for the enzyme eluted from the immunoaffinity column is in high salt and ethylene glycol. We plan to use spin columns to exchange the buffers before assay for the activity in a reproducible manner.

Task 3. Study of the response of the pol δ promoter in normal and breast cancer cell lines.

As mentioned in Task 1 , there may a link between the increase of p53 level and the decrease in polymerase δ message level after DNA damage. Taking advantage of the

luciferase assay system due to the high sensitivity and low background in mammalian cells, we transfected luciferase reporter plasmid driven by POLD1 promoter into different cell lines and measured the relative pol δ p125 promoter activity in the cells. The results are shown in Figure 4. Saos2, HeLa, MCF7 and MDA-MB 231 cells were transfected with pGL2-δ (-1758) alone or together with pC53-SN3. As can be seen the human breast cell lines expressed very low level of reporter activity compared to human Saos2 cell line or HeLa cell line. These studies also suggested that the expression of pol p125 subunit may be governed by different up-regulated or down-regulated molecular mechanisms.

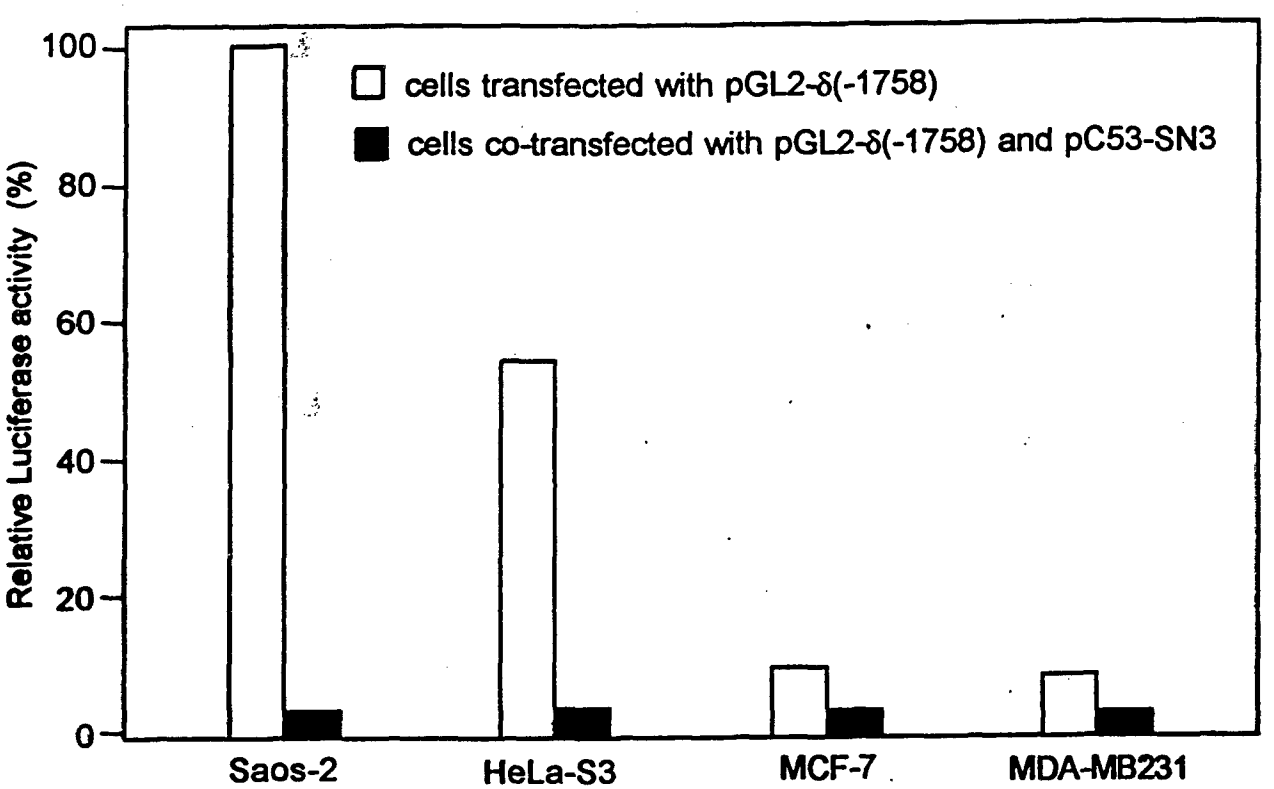

Fig. 4. Relative Pol δ p125 promoter activities in human cells and its down-regulation by p53 . Human pC53-SN3 mammalian expression vector was a gift from Dr. Vogelstein. PGL2 δ (-1758) was constructed by linking the 5' flanking region of POLD1 with pGL2-basic luciferase expression vector (promoter). The cells were grown to a density of 80% confluency, harvested by trypsinization. Then the cells were pelleted by centrifugation at 1,500 rpm, and washed once with phosphate buffered saline. The transient expression of plasmid DNA in the human cells were carried out by electroporation. After pelleted by centrifugation, about 2X 10^6 cells were resuspended in

500 ml of a mixture of serum-free F12 and Dulbecco's modified Eagle's medium (DMEM) containing 20 mg of luciferase plasmid DNA and 5 mg of ß-galactosidase plasmid DNA, then transferred into a electroporation cuvette . The electroporator was charged for at least 3 min before pulsing the samples. The cells were then incubated on ice for 5 min, resuspended in 10% FBS growth medium, transferred to a 6 well tissue culture cluster grown in 37°C incubator and assayed for luciferase activity assay. The luminant light generated by luciferase was measured about 30 seconds immediately after mixing 100 ml of cell extract and luciferase assay agent solution (Promega) by liquid scintillation analyzer (Packard TRI-CARB) using its proton measuring protocol. The final count values (cpm) were normalized by ß -galactosidase activity which was used as an internal control.

To investigate the mechanism underlying a correlation between p53 and POLD1 expression, we studied the regulation of p53 on POLD1 gene transcription.

A 1.8 Kb POLD1 promoter was cloned and shown to have 32% promoter activity compared with that of the SV40 promoter. This promoter is TATA-less and has no CCAAT site. The promoter is GC rich with 64% GC content. It was shown that Sp1 and Sp3 but not Sp2 binds specifically to two 11 bp repeat sequences and activates the promoter activity (*Zhao and Chang*, 1997). Inspection of its sequence identified several putative binding sites for transcription factors such as Ap1, ATP/CREB, E2F and p53.

During the past year we have made rapid progress in these studies. The cell systems we proposed to use are functioning well in our hands, and the preliminary data obtained below provides strong support for the feasibility as well as the potential significance of the studies. The data show that p53 is able to inhibit the transcription of the pol δ gene, and we have made a positive identification of the region of the POLD1 promoter that is the target of p53. The preliminary analyses point to a mechanism whereby p53 exerts a transcriptional repression of the pol δ gene promoter. This is consistent with other reports that p53 can inhibit the expression of other genes that are involved in promoting cell growth and cell cycle progression, *e.g.*, PCNA (Mercer et al., 1991; Jackson et al., 1994) and cyclin A (, Desdouets et al., 1996). p53 also exerts transcriptional repression of viral genes, *e.g.*, SV-40 (Jackson et al, 1993). There is also evidence that the actions of p53 in apoptosis may also involve transcriptional repression of certain genes, *e.g.*, c-fos (Kley et al., 1992; Ryan and Vousden, 1998), MAP4 (Murphy et al., 1996).

Demonstration of the regulation of pol δ mRNA levels by p53.

We have set up the tet-off cell (H24-wtp53-14) system in which p53 expression can be tightly regulated by the levels of tetracycline in the culture media. Using this cell

line we have shown that expression of p53 results in a significant reduction of pol δ mRNA levels. The results of one such experiment is shown in Fig.5. The cell line behaves in our hands according to published reports, in that the p53 expression is tightly regulated as shown by Western blotting. Concurrent Northern blotting for pol δ mRNA shows a parallel decrease in pol δ mRNA. These results provide a direct confirmation that pol δ mRNA levels are decreased in parallel with the increased expression of p53 in a cellular context.

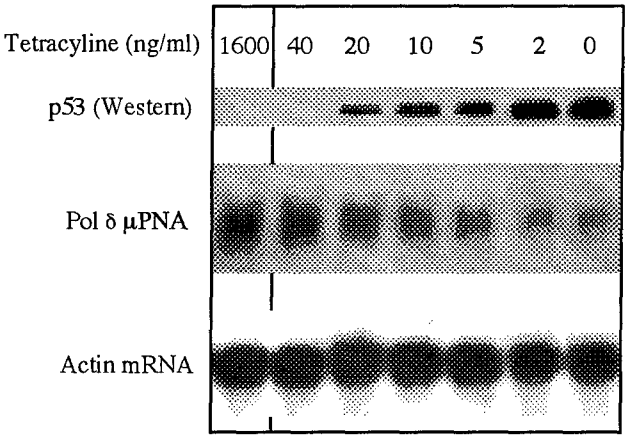

Fig.5. Effect of p53 expression on Pol δ mRNA levels.
H24-wtp53-14 cells were grown in P100 dishes in the presence of the indicated levels of tetracycline. The cells were harvested and lysed 24 hr after the addition of tetracyline. Cell extracts were analyzed for the expression of p53 by Western blotting. Total mRNA was isolated from the cells using RNA-STAT 60 (Tel-Test Inc) and subjected to Northern blotting using a probes for pol δ. The membranes were stripped and reprobed for γ-actin.

Repression of the activity of the POLD1 promoter by p53.

The effects of p53 expression on the activity of the POLD1 promoter were tested using the 1.8 kb-luciferase POLD1 promoter construct in SAOS-2 cells which do not express p53. The POLD1 promoter was co-transfected with different amounts of a p53 expression plasmid (pCMV-53). A number of experiments were performed, which showed that the POLD1 promoter activity is strongly repressed by co-expression of the p53 expression plasmid in a dose-dependent manner (Fig.6).

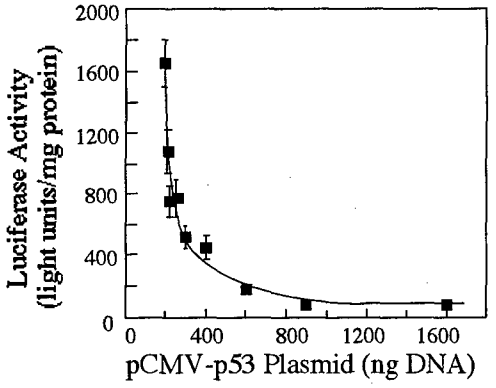

Fig.6. Repression of the POLD1 promoter activity by co-transfection with an expression plasmid for p53.
SAOS-2 cells were co-transfected with a fixed amount of the POLD1 promoter fused to a luciferase reporter and increasing amounts of the pCMV-p53 plasmid. Cells were harvested and analyzed for luciferase activity. Results are shown as arbitrary light units per mg of cell extract protein. Error bars show the mean of four independent experiments.

Identification of the P4 site in the POLD1 promoter as the likely p53 binding site.

In preliminary studies using deletion constructs of the promoter we were able to eliminate three of the 5 putative p53 binding sites as shown in Fig.7, as the deletion construct in which P1, P2 and P3 were removed showed the same degree of repression by p53 as the full length promoter.

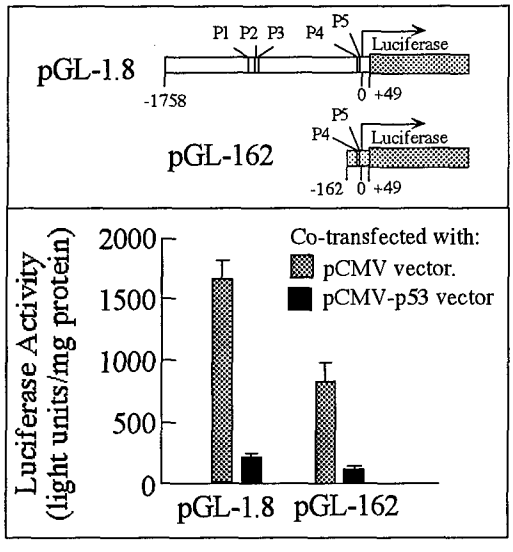

Fig.7. Effect of deletion mutagenesis of the POLD1 promoter on its repression by p53. The top panel shows a map of the POLD1 promoter fused to the luciferase reporter gene in the pGL-basic vector. The positions of the putative p53 sites are shown as P1-P5. pGL-162 is the deletion construct in which the region from -1726 to -162 was deleted. The POLD1 constructs were co-transfected with a control pCMV vector or with a pCMV-p53 vector. Cells were collected 24 hr after transfection and luciferase activities were determined (bottom panel). Data are shown as arbitrary light units per mg of cell lysate protein. Data are for experiments done in triplicate. For both constructs the co-expression of p53 led to a 87-88% repression of POLD1 promoter activity.

In preliminary studies using gel mobility shift assays we tested ds oligonucleotides corresponding to all five sites (P1-P5). The results showed that only the oligo with the P4 site (-90 to –65, see Fig.7) exhibited a gel shift with p53. We have carried out additional gel shift assays, and have further confirmed that the P4 site interacts with p53 (Fig.8). To further confirm that the gel shifts that were observed were due to an interaction with p53, the gel shift assays were performed in the presence of different amounts of a consensus p53 motif, a mutated p53 motif, and unlabeled P4 oligonucleotide. The results are shown in Fig.8.

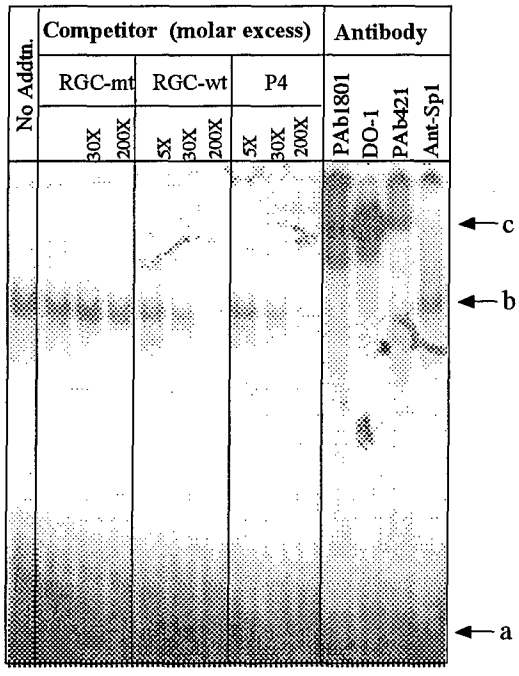

Fig. 8. Gel shift assays of the P4 element by p53.
A labeled probe corresponding to the –90 to –65 sequence of the POLD1 promoter was used. The probe was incubated with recombinant p53 and then subjected to acrylamide gel electrophoresis and autoradiography (extreme left lane). The next three panels show the competition with unlabeled oligos. The last panel shows the effects of adding antibodies to p53. (**a**, unlabeled P4 oligo; **b**, shift in the presence of p53; **c**, supershift in the presence of p53 and antibody to p53). For these experiments p53 was overexpressed in Sf9 cells and purified to homogeneity by chromatography on Q-Sepharose and immunoaffinity chromatography.

The experiment shows the effects of competition with the known ribosomal gene cluster (RGC) consensus p53 sequence (RGC-wt) and a mutant (RGC-mt) control which does not bind to p53 (Kern et al., 1991). Only the wt p53, or the unlabelled P4 probe, were able to compete. In parallel, gel shift assays in which three different antibodies to p53 (PAB1801, DO-1 and PA421) were added showed that there was a supershift, *i.e.*, the observed shift is confirmed to be due to the binding of p53. An antibody to Sp1 did not cause a shift. To further confirm the gel shift results, we are in the process of performing *in vitro* DNaseI footprinting experiments to identify precisely the p53 binding regions on both sense and anti-sense strands of the promoter.

Technical objective 2 The multi-protein complexes of pol δ from normal and breast cancer cell lines and tissues will be studied to determine if a) they display functional defects and b) to determine if they exhibit altered behavior in terms of protein-protein interactions.

Task 4. Comparison of DNA replication functions of multi-protein pol δ complexes of normal and breast cancer cells.

We have an interesting finding this year. Dr. Krucher showed that the product of the retinoblstoma tumor suppressor gene (pRb) interacted with the catalytic subunit of DNA polymerase δ (Fig. 9) . Using GST-pRb constructs, we have shown that p125 from MCF10A cell extracts associates with pRb. The pRb that associated with GST-p125 was found to be largely hypophosphorylated. Co-immunoprecipitation experiments using cell cycle synchronized cells showed that p125 and pRb form a complex predominantly in G1, the phase during which pRb is mainly hypophosphorylated. In vitro phosphorylation of GST-pRb by the cdks reduced the ability of GST-pRb to associate with p125. Finally, the addition of the LXCXE containing protein large T antigen to GST-pRb blocks the ability of the p125 to associate with pRb,suggesting that it is through a similar amino acid sequence by which p125 interacts with pRb

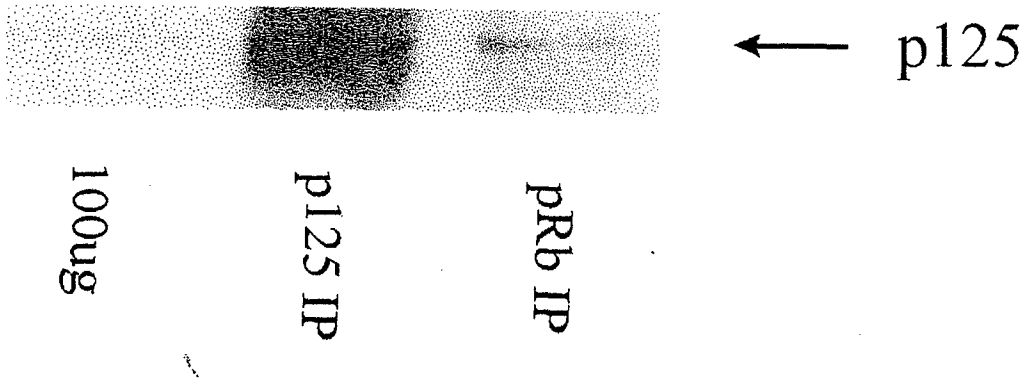

Fig 9. Coimmunoprecipitation of p125 with pRb in MCF 10A cells. Cell lysates containing 0.5-1.0mg were normalized to the same volume (approximate protein concentration 1 mg/ml) and following a 1 hour preclearing with Staphylococcus protein A or protein G Sepharose beads (Sigma), immunoprecipitated for 1.5 to 2 hours with primary antibody at 4 C. Next , 100 ul of a 1:1 slurry of protein A or protein G beads was added and the precipitations were incubated an additional hour. After three washes

with kinase buffer, immunoprecipitates were analyzed by SDS- PAGE. Lane1. Cell lysates 100 μg. Lane 2. 100 μg lysate immunoprecipitated with p125 monoclonal antibody Lane 3. 100 μg of lysate immunoprecipitated with pRb antibody. The gel was transferred and immunoblotted with p125 antibody.

Task 5. Study of protein-protein interactions within complexes of pol δ .

This is well underway. Four papers result from this work :

1) Characterization of the p125 Subunit of Human DNA Polymeraseδ and Its Deletion Mutants" J. B. C. 273, 9561-9569 (1998)

2) Identification of DNA Replication and Cell Cycle Proteins that Interact with PCNA. Nucleic Acids Research 25, 5041-5046 (1998)

3) Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA Polymerase δ. J.Biol. Chem. 274, 26647-26653(1999)

4) Purification and Characterization of a Holoenzyme-like Form of DNA Polymerase δ: identification of p68 as a subunit of mammalian DNA polymerase δ. Submitted to J. Biol. Chem.

We have developed a native gel electrophoresis technique. This coupled with PCNA overlay technique will be very powerful to study protein -protein interaction between pol δ and PCNA complexes.

We have repeated several times the immunoblot analysis of components of pol δ complex isolated from our polymeraseδ immunoaffinity column . It was found, for example, that cyclin D1 is only found in the phenotypically normal MCF10 A cell lines and not in the cancerous MCF7 and MDA MB231 cell lines. It has been reported by Xiong et al.(1993a,b) that the cyclin D1 gene is present in a rearranged or amplified form in 20% of all breast tumors (Xiong et al.,1993a). Thus, our biochemical data agreed with their finding. These results are surprising, but are consistent with a redistribution of PCNA from the replication complex to the cyclin complexes as has been proposed by Xiong et al., (1993b). A sequestering of PCNA by the D cyclins could explain their absence in the immunoaffinity purified pol δ.

p21 was found in the complex from the normal cell line (MCF 10A) but was absent in MDA MB 231 complexes and was lost from the MCF 7 complex after gel filtration. Our conclusion is that p21 is dissociated from the complex on gel filtration.

More recently a new gene and protein called p16, which has been shown to be damaged in a large proportion of many types of cancer has been identified. (Alcorta et al., 1996) Therefore, the immunoblot will also performed in the coming year. This p16 antibody has been ordered from Santa Cruz Biotechnology Inc.

Technical Objective 3. The ability of extracts of the breast cancer cell lines to carry out nucleotide excision repair will be compared to that of normal breast cell lines using an in vitro repair assay.

Task 6. Assay of repair activities of nuclear extracts of normal and breast cancer cell lines.

Not yet initiated

Task 7. Study of repair synthesis of multi-protein polδ complexes from normal and breast cancer cells.

Not yet initiated

Technical objective 4. Normal and breast cancer cell lines and tissues will be s creened for genetic alterations in the pol δ and PCNA gene

Task 8. RT-PCR and genomic PCR analyses of 3' to 5' exonuclease and N2 domains of pol δ .

In order to detect the mutation of the POLD1 gene, we have isolated genomic DNA from twenty-three breast cancer tissues and three breast cancer lines. After purifying the genomic DNA, we cut it with EcoR I (an EcoR I site locates at position 97 of p125 cDNA) and run 1% agarose gel. As shown in Fig. 10, the band patterns of the lines 1, 8, and 23 are different from other lines. The difference may result from the mutation of POLD1 gene in breast cancer.

To further detect the mutations of the POLD1 gene, we will use a new technique NIRCA, non-isotopic RNase Cleavage Assay. In this procedure, mutations are detected by ribonuclease cleavage of both strands of duplex RNA targets containing base-pair mismatches. To scan for mutations with NIRCA, we are amplifying a length of 3 kb

POLD1 genomic DNA fragment, covering the 3' to 5' exonuclease domain and PCNA binding regions from different breast cancer tissues and breast cancer cell lines. In a secondary PCR procedure, the first round of PCR products will be amplified into four different groups of PCR products, which contain N2, N4, Exo I, Exo II and Exo III regions respectively. These targets are amplified by PCR using primers with 22 base T7 promoter on the 5' end. The length of these crude PCR products is about 0.75 kb. They will then be transcribed in vitro using phage RNA polymerase and hybridized to form double-stranded RNA. Mutations in the test regions result in base-pair mismatches when complementary reference (wild type) and mutant transcripts are hybridized. After hybridization, the duplex RNA targets will be treated with a mixture of RNases capable of cleaving base-pair mismatches on both strands. Finally, the double-stranded cleavage products will be separated on a native agarose gel and detected by ethidium bromide staining under UV light. The NIRCA-detected mutants will be further analyzed by DNA sequencing.

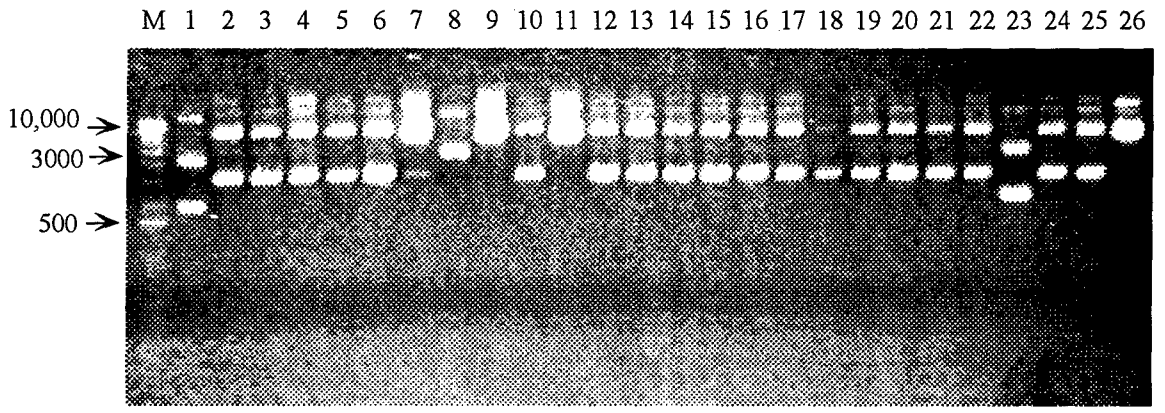

Figure 10: Restriction enzyme pattern of EcoR I of genomic DNA from breast cancer tissues and cell lines.

Line 1: Breast cancer cell line Hs578T,
Line 2: Breast cancer cell line Hs578Bst,
Line 3: Breast cancer cell line MCF-7,
Line 4: Normal breast cell line MCF-10A,
Line 5: Normal breast tissue from Breast Cancer Tissue Bank of University of Michigan,
Lines 6- 26: Patient-derived sample of human breast cancer from Breast Cancer Tissue Bank , University of Michigan.

Task 9. Analysis of functional properties of mutations found in breast cancer cell lines and tissues.

Not yet initiated

Task 10. PCR analyses of the promoter region of pol δ in breast cancer cell lines and tissues.

Using NIRCA and DNA sequencing stated in Task 8, we are also investigating the mutants of the POLD1 promoter. We have amplified a length of 2 kb of the POLD1 genomic DNA fragment covering the full length 1.8 kb from different breast cancer tissues and different breast cancer cell lines. In a secondary PCR procedure, the first round of PCR products will be amplified during this grant period into three different groups of PCR products. The targets will be amplified using primers with 22 base T7 promoter on the 5' ends. The lengths of these secondary crude PCR products will be about 0.7 kb. If we find some interesting mutated sites in the POLD1 promoter, we will introduce these mutations into the pGL2 delta reporter plasmid. The function of the mutated promoter will be compared to that of the normal promoter.

Task 11. RT-PCR and genomic PCR analyses of human PCNA.

Not yet initiated.

7. Key Research Accomplishments

1) We have used breast cancer cell lines to probe for changes in proteins associated with polymerase δ complexes isolated by immunoaffinity chromatography.
2) In the case of the transformed breast cell lines, significant differences were observed in that several of the protein ligands were not present in the affinity purified fraction consistent with the findings of other investigators. Of note is that p21 was found in the complex from normal cell lines but absent in breast tumor cell lines we studied.
3). There is a correlation between accumulated p53 and POLD1 mRNA inhibition in cellular response to DNA damaging agents.

4). The protein levels of pol δ catalytic subunit and p53 are also correlated in response to DNA damaging agents. As the protein level of p53 does up, the pol δ protein level goes down .

6). We are the first to show that the tumor suppressor Rb interacts with pol δ.

8. Reportable Outcomes

Manuscripts.

1. Wu S.M., Zhang, P., Zeng, X.R., Zhang, S.J., Mo, J., Li, B.Q., and Lee, M.Y.W.T. Characterization of the p125 subunit of Human DNA polymerase delta and Its Delection Mutants" J. B. C. 273, 9561-9569 (1998)
2. Loor, G., Zhang, S.J., Zhang, P., Toomey, N.L., and Lee, M.Y.W.T. Identification of Dna replication and cell cycle proteins that interact with PCNA Nucleic Acids Research 25, 5041-5046 (1998)
3. Zhang, P., Mo, J., Perez, A., Leon, A., Liu, L., Mazloum, N., Xu, H., and Lee, M.Y.W.T. Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA polymerase delta. J.Biol. Chem. 274, 26647-26653(1999)
4. Mo, J., Leon, A., Mazloum, N., Liu, L., and Lee, M.Y.W.T. Purification and characterization of a holoenzyme-like form of DNA polymerase delta: identification of p68 as a subunit of mammalian DNA polymerase delta. Submitted to J. Biol. Chem.
5. Krucher, N., Mazloum, N., Tamrakar,S., Ludlow, J., and Lee, M.Y.W.T. Interaction of pRB with DNA polymerase delta catalytic subunit (p125) in preparation

Abstracts.

1) Li, B.Q., Chen, X., and Lee, M.Y.W.T. Transcriptional repression of DNA polymerase delta catalytic subunit Gene (POLD1) by p53 tumor suppressor. Abstract #443 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999
2) Liu, L., Rodriquez-Belmonte, E., and Lee, M.Y.W.T. Cloning the putative human DNA polymerase delta thilrd subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

3) Mazloum, N., lee, MY.W.T. , and Zhang, P. The Examinatin of protein-protein interaction between the catalytic subunit of DNA polymerase delta (p125) and the proliferating cell nuclear Antigen (PCNA) Abstract #343 _mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

4) Mazloum , N., Krucher, N., Liu, L., and Lee, M.Y.W.T. Physical Association between DNA polymerase delta and P21. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999

5) Liu, L., Rodriquez-Belmonte, E., and Lee, M.Y.W.T. DNA polymerase delta holoenzyme: how many subunits are there? Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999

6) Gupte, R., Mo, J., Liu, L., Mazloum, N., and Lee, M.Y.W.T. Further Studies of the Immunoaffinity purified Pol delta complex Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999

7) Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and Lee, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase delta. Eukaryotic DNA Replication Meeting Cold Spring Harbor 1999

8) Xu, H., and Lee, M.Y.W.T. Analyzes of POLD1 gene mutation and study of its transcriptional regulation in Breast Cancer Cells. Breast Cancer Research Program Era of Hope 2000

Oral Presentations

1. Liu, L., Rodriquez-Belmonte, E., and Lee, M.Y.W.T. Cloning the putative human DNA polymerase delta thilrd subunit, which binds to the small subunit p50 and PCNA Abstract #337 Molecular mechanisms in DNA Replication and Recombination . Keystone Symposia 1999

2. Zhang, P., Liu, L., Mo, J., Mazloum, N., Xu, H., and Lee, M.Y.W.T. Direct Interaction of PCNA with p125, the catalytic subunit of mammalian DNA polymerase delta. Eukaryotic DNA Replication Meeting Cold Spring Harbor

9. Conclusion.

DNA polymerase δ, the principal replicative DNA polymerase in mammalian system is responsible for the elongation of leading strand and for the completion of Okazaki fragment on lagging strand synthesis. We have used an immunoaffinity column to the

p125 subunit and co-immunoprecipitation experiments to show that polymeraseδ is linked to cell cycle and repair enzymes. We have linked polymerase δ to p21, and to the tumor suppressor genes p53 and Rb. These are of significance for the finding that p53 represses polymerase δ is a novel route for its regulation of DNA replication after being induced by DNA damaging agents.

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11. Appendices.

1) Wu S.M., Zhang, P., Zeng, X.R., Zhang, S.J., Mo, J., Li, B.Q., and Lee, M.Y.W.T. Characterization of the p125 subunit of Human DNA polymerase delta and Its Delection Mutants" J. B. C. 273, 9561-9569 (1998)

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Characterization of the p125 Subunit of Human DNA Polymerase δ and Its Deletion Mutants

INTERACTION WITH CYCLIN-DEPENDENT KINASE-CYCLINS*

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The catalytic subunit of human DNA polymerase (pol) δ was overexpressed in an active, soluble form by the use of a baculovirus system in insect cells. The recombinant enzyme was separated from endogenous DNA polymerases by phosphocellulose, Mono Q-Sepharose, and single-stranded DNA-cellulose chromatography. Recombinant DNA pol δ was also purified by immunoaffinity chromatography. The enzymatic properties of the purified catalytic subunit were characterized. The enzyme was active and possessed both DNA polymerase and associated 3′ to 5′ exonuclease activities. NH_2-terminal deletion mutants retained polymerase activity, whereas the core and COOH-terminal deletion mutants were devoid of any measurable activities. Coinfection of Sf9 cells with recombinant baculovirus vectors for pol δ and cyclin-dependent kinase (cdk)-cyclins followed by metabolic labeling with $^{32}P_i$ showed that the recombinant catalytic subunit of pol δ could be hyperphosphorylated by G_1 phase-specific cdk-cyclins. When cdk2 was coexpressed with pol δ in Sf9 cells, pol δ was found to coimmunoprecipitate with antibodies against cdk2. Experiments with deletion mutants of pol δ showed that the NH_2-terminal region was essential for this interaction. Coimmunoprecipitation and Western blot experiments in Molt 4 cells confirmed the interaction *in vivo*. Preliminary experiments showed that phosphorylation of the catalytic subunit of pol δ by cdk2-cyclins had little or no effect on the specific activity of the enzyme.

DNA polymerase (pol)[1] δ is the central enzyme in eukaryotic DNA replication (1) and also serves an important role in DNA repair (2). Isolation of the calf thymus (3) and human (4) enzymes has shown that it consists of at least two core subunits of 125 and 50 kDa. The hallmarks of this polymerase are that it has an intrinsic 3′ to 5′ exonuclease activity, distinguishing it from pol α and pol β. The 125-kDa subunit of human pol δ (p125) has been identified as the catalytic subunit (4). Pol δ is a member of a family of DNA polymerases which includes DNA polymerase α, pol ε, the herpesvirus DNA polymerases, and bacteriophage T4 polymerase (5, 6). Examination of the regions of conserved sequence has led to the identification of domains that are potentially required for DNA interaction, deoxynucleotide interaction, as well as the 3′ to 5′ exonuclease activity of pol δ (7). In addition, there are several regions in the NH_2 and COOH termini which are conserved among human pol δ, yeast pol δ, and yeast and human pol ε (5, 7).

Studies of the replication of SV40 DNA *in vitro* have led to the identification of a number of accessory proteins, which, together with pol δ, are required for the formation of a replication complex at the replication fork. These include PCNA, which functions as a sliding clamp and enhances the processivity of pol δ, consistent with its role as the leading strand polymerase (8). Although there have been some mutagenesis studies of the yeast pol δ (9), little has been done with human or mammalian pol δ, largely because of the lack of a suitable expression system. To facilitate structure-function studies of pol δ, it is desirable to have an expression system for the production of the recombinant protein. The expression of the human pol δ catalytic subunit has been achieved in mammalian cells using a vaccinia virus vector (10). In this study we report the expression of p125 in Sf9 cells using a baculovirus vector as well as methods for separating the recombinant protein from endogenous DNA polymerases in baculovirus-infected Sf9 cells. Deletion mutants of p125 were also characterized to investigate the domain structure of pol δ. In addition, we have obtained novel evidence that pol δ p125 is phosphorylated by the cyclin-dependent kinase (cdk)-cyclin complexes and also can be coimmunoprecipitated with cdk2 when they are coexpressed in Sf9 insect cells. The interaction of pol δ with the cyclins and cdks was also confirmed by coimmunoprecipitation and Western blot experiments in Molt 4 cells. Preliminary experiments showed that phosphorylation has moderate or little effect on the activity of the catalytic subunit.

EXPERIMENTAL PROCEDURES

Materials—Sf9 cells were purchased from Invitrogen and were maintained at 27 °C in TNM-FH insect medium supplemented with 10% fetal calf serum and 50 μg/ml gentamycin. Cells were propagated both as adherent monolayers and as nonadherent suspension cultures. These cells were used as the hosts for the propagation of wild type *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) and recombinant baculoviruses. Cyclin and cdk recombinant baculoviruses were gifts of Dr. Charles Sherr (St. Jude's Hospital, Memphis, TN). BaculoGold™-linearized baculovirus DNA was purchased from Pharmingen. The baculovirus transfer vector P2bac was purchased from Invitrogen. Plasmid pALTER-1 was purchased from Promega.

Construction and Screening of Recombinant Baculoviruses—The coding sequence of pol δ which was used in these studies was derived from the cDNA originally isolated by Yang *et al.* (7). This coding sequence was inserted into the pALTER vector and corrected by site-directed

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[1] The abbreviations used are: pol, polymerase; p125, 125-kDa subunit of human pol δ; cdk, cyclin-dependent kinase; AcMNPV, *A. californica* multiple nuclear polyhedrosis virus; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen.

mutagenesis so that His-119, Asn-173, and Gly-776 were mutated to Arg-119, Ser-173, and Arg-776 to conform to the genomic sequence (10, 11). The plasmid pALTER-pol δ containing the corrected full-length pol δ coding sequence (3.5 kilobases) was excised from the pALTER plasmid by *Bam*HI/*Hin*dIII digestion, gel purified, and inserted into *Bam*HI/*Hin*dIII-digested baculovirus transfer vector p2bac. The recombinant p2bac plasmids were cotransfected into Sf9 cells with wild type baculovirus DNA according to Ausubel *et al.* (12). Wild type BaculoGold™-linearized AcMNPV DNA (1 μg), recombinant plasmid DNA (3 μg), cationic liposome solution (25 μl), and 1 ml of Grace's insect medium containing no supplements were mixed by vortexing for 10–15 s and incubated at room temperature for 15 min. The transfection mixture was then layered onto Sf9 cells growing on 60-mm plates. After 4 days at 27 °C, the medium was aspirated and analyzed for virus production by plaque assay. The recombinant baculoviruses were identified as occlusion-negative plaques with a dissecting microscope. Because the BaculoGold™-linearized virus DNA contains a lethal deletion and a *lacZ* gene, the small portion of nonrecombinant virus plaques stained blue on 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside plates, whereas all recombinants produced colorless plaques on these plates. After three rounds of plaque purification, pure recombinant baculoviruses were obtained. Occlusion-negative viral stocks were prepared from the final supernatants, titered, and stored at 4 °C. Deletion mutants of pol δ were constructed as described in Ref. 13.

Infection of Sf9 Cells with Recombinant Baculovirus and Preparation of Cell Extracts—Recombinant viral stocks (0.5 ml) were added to a multiplicity of infection between 5 and 10 for the infection of log phase Sf9 cells for 1 h. The inoculum was then removed from the plates, and 8 ml of fresh complete TNM-FH insect medium was added. The infected Sf9 cells were allowed to grow for 2 days at 27 °C and were harvested 48 h postinfection. Cells were harvested from 80 100-mm plates and collected by centrifugation. The cell pellets were washed twice with ice-cold phosphate-buffered saline, pH 7.4. Subsequent manipulations were carried out at 4 °C. The cells from 80 plates (about 8×10^8 cells) were suspended in 5-cell pellet volumes (50 ml) of lysis buffer (40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.1 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine-HCl). Cells were disrupted by passage through a French press at 1,000 p.s.i. The lysate was centrifuged at $27,000 \times g$ for 30 min. The supernatant was removed and saved as the soluble extract, and the pellet was suspended in 20 ml of lysis buffer plus 0.5 M NaCl and sonicated three times for 20 s each at 50 watts on ice. The extract was again centrifuged at $27,000 \times g$ for 30 min, and the supernatant was designated as the high salt-solubilized fraction. Protein concentrations of the first and second extracts were 12 and 9 mg/ml, respectively. The pellet was then dissolved in 1 ml of 8 M urea. The two fractions (low and high salt extracts) were then combined and dialyzed against TGEED buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, I mM dithiothreitol).

Phosphocellulose Chromatography—The dialyzed lysates were loaded onto a phosphocellulose column (5 × 7 cm) equilibrated in TGEED buffer. The column was eluted with a linear gradient of 50–1 M NaCl in TGEED buffer in a total volume of 2 liters. Fractions of 10 ml each were collected and assayed for DNA polymerase activity. Western blots were also performed using 38B5, a monoclonal antibody against the COOH-terminal region of pol δ (2, 14).

HPLC—The combined fractions from the phosphocellulose column which contained recombinant pol δ p125 were dialyzed against TGEED buffer, pH 7.8, passed through an 0.45-μm syringe filter, and injected onto a Mono Q HR 5/5 column. The enzyme was eluted with a linear gradient of 0–1 M NaCl for 20 min at 1 ml/min.

Single-stranded DNA-cellulose Chromatography—Fractions from the Mono Q column were dialyzed against HEPES buffer (20 mM HEPES, 5 mM $MgCl_2$, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5) and were then loaded onto a single-stranded DNA-cellulose column (0.5 × 6 cm) equilibrated with HEPES buffer. The column was washed with the same buffer, and a gradient of 50–500 mM NaCl in a total volume of 40 ml was applied. Fractions of 1 ml were collected and analyzed by SDS-PAGE, Western blotting, and assays for pol δ activity.

Immunoaffinity Chromatography—Monoclonal antibody 78F5 was coupled onto AvidChrom hydrazide (Sigma) as described by Jiang *et al.* (14). The column (1 × 10 cm) was equilibrated with TGEE buffer (50 mM Tris-HCl, 0.1 mM EGTA, 0.5 mM EDTA, 10% glycerol, pH 7.8). The column was washed with the same buffer containing 50 mM NaCl, and pol δ was eluted with 0.2 M NaCl in TGEE buffer. Fractions of 1 ml were collected and analyzed as described above.

DNA Polymerase Assays—Sparsely primed poly(dA)·oligo(dT) was used as the template as described by Lee *et al.* (3). The standard reaction for the poly(dA)·oligo(dT) assay contained 0.25 optical density units/ml poly(dA)·oligo(dT) (20:1), 200 μg/ml bovine serum albumin, 5% glycerol, 10 mM $MgCl_2$, 25 mM HEPES, pH 6.0, 100 cpm/pmol [^{3}H]TTP, and 0.2–0.4 unit of pol δ in the presence or absence of 0.2 μg of PCNA in a total volume of 100 μl. Reaction mixtures were incubated for 60 min at 37 °C and were terminated by spotting onto DE81 papers that were then washed four times with 0.3 M ammonium formate, pH 7.8, once with 95% ethanol, and counted as described previously (4).

Assay for 3′ to 5′ Exonuclease Activity—The assay was performed by measuring the release of [^{3}H]dTMP from [^{3}H]dT_{50} as described previously (3). The assay contained 2 μM [^{3}H]dT_{50} (200–300 cpm/pmol), 25 mM HEPES buffer, pH 7.4, 5 μg of bovine serum albumin, 5 mM $MgCl_2$, and 0.2–0.4 unit of pol δ in a total volume of 60 μl. Reaction mixtures were incubated for 30 min at 37 °C and were terminated by spotting 20 μl onto DE81 filter papers. Filters were washed four times with 0.3 M ammonium formate, pH 7.8, and once with 95% ethanol and counted as described previously (3).

Western Blot Analysis—The recombinant proteins expressed in Sf9 cells infected with recombinant baculoviruses were analyzed by Western blotting with pol δ monoclonal antibody 38B5 (2, 14). Extracts of Sf9 cells prepared as described above were subjected to SDS-PAGE in 5–15% gradient gels that were then transferred to nitrocellulose membranes. Prestained protein standards (Sigma) were used as molecular weight markers and also to provide visual confirmation of efficient transfer. The nitrocellulose blots were blocked with 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, for 1 h at room temperature. The blots were then incubated with monoclonal antibody against pol δ for 12 h at 25 °C. After three 10-min washes in 50 mM Tris-HCl, pH 7.5, 0.15 M NaCl, the blots were incubated with biotinylated sheep anti-mouse immunoglobulin for 1 h at 27 °C followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide.

Coinfection of Sf9 Cells with Pol δ, Cyclins, and Cdks and $^{32}P_i$ Labeling—Sf9 cells (10^7) were grown to exponential stage. Pol δ, cyclin, and cdk recombinant baculoviruses (0.5 ml) were added as indicated. The cells were infected at room temperature for 1 h. The recombinant baculoviruses were removed, replaced with growth medium, and the cells were grown for an additional 2 days at 27 °C before labeling with $^{32}P_i$. Infected Sf9 cells were transferred into a 15-ml tube for $^{32}P_i$ labeling. After centrifugation and removal of growth medium, the cells were resuspended in 2 ml of fresh phosphate-free medium containing 200 μCi of $^{32}P_i$ (specific activity 3,000 Ci/mmol) and incubated at 37 °C for 2 h. The cells were centrifuged at $3,000 \times g$ for 5 min. The supernatant was removed, and the cells were washed twice with phosphate-buffered saline. The cells were sonicated for 30 s in 40 mM Tris-HCl, pH 7.8, 0.25 M sucrose, 0.5 M NaCl, 0.1% Nonidet P-40, 0.1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine-HCl. The crude cell extracts were transferred to microtubes and centrifuged at $15,000 \times g$ for 30 min. About 20 mg of total protein was used for immunoprecipitation in the presence of 20 μg of 78F5 pol δ monoclonal antibody (2, 14) and 40 μl of protein A-Sepharose slurry at 4 °C overnight. The Sepharose beads were washed twice with sonication buffer and boiled for 5 min in 50 μl of SDS sample buffer. The proteins released from the beads were then subjected to SDS-PAGE and autoradiography.

Immunoprecipitation and immunoblotting of Molt 4 Cells with Pol δ and Members of the Cyclin and Cdk—4×10^7 exponentially growing Molt 4 cells were prepared and lysed with 300 μl of Nonidet P-40 buffer (50 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 150 mM NaCl, and 1% Nonidet P-40). The lysates were precleaned with protein A beads (50 μl of a 10% suspension) by rotating at 4 °C for 30 min. The supernantants were removed by centrifugation and transferred to a fresh tube. The antibody used for immunoprecipitation was then added in the presence of 50 μl of fresh protein A beads and incubated at 4 °C for 1 h. Anti-pol δ monoclonal antibody (20 μg), PCNA monoclonal antibody (20 μg), anti-cyclin E and A antibodies (100 μl of hybridoma cell supernatant), and anti-cdk2 polyclonal antibody (2 μl) were used for the experiments. The extracts were then centrifuged and washed with Nonidet P-40 buffer three times. After SDS-PAGE, the separated proteins were transferred to a nitrocellulose membrane and Western blotted with antibodies to cdk2, cdk5, or pol δ.

RESULTS

Expression of Pol δ p125—The expression of human pol δ in Sf9 cells infected with recombinant baculovirus was analyzed

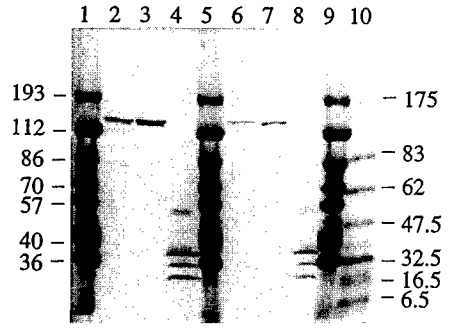

FIG. 1. **Immunoblot of extracts of Sf9 cells infected with recombinant baculovirus.** Extracts of Sf9 cells infected with recombinant baculoviruses were prepared as described under "Experimental Procedures." The cells were disrupted and extracted in 50 ml of lysis buffer containing 0.1 M NaCl and then with 20 ml of lysis buffer containing 0.5 M NaCl. The pellet was then dissolved in 1 ml of 8 M urea. These three extracts (60 or 30 μg of protein/*lane*) were then analyzed by SDS-PAGE (5–15% acrylamide). Western blotting was performed using monoclonal antibody 38B5 against human pol δ. *Lanes 1, 5,* and *9* are high molecular weight standards as marked; *lanes 2–4* are 60 μg of the 0.1 M NaCl, 0.5 M NaCl, and 8 M urea extracts, respectively. *Lanes 6–8,* same as *lanes 2–4* but with 30 μg of protein/lane; *lane 10,* low molecular weight protein standards as marked.

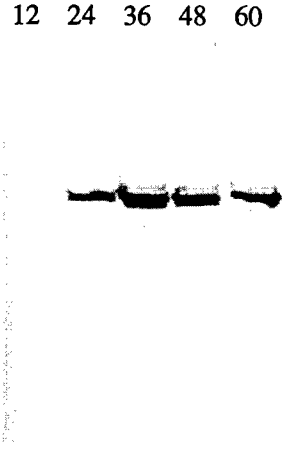

FIG. 2. **Time course of pol δ expression in Sf9 cells.** Sf9 cells infected with recombinant virus were harvested at 12, 24, 36, 48, and 60 h after infection. The cells were lysed and extracted as described under "Experimental Procedures." The 0.1 M and 0.5 M NaCl extracts were combined and analyzed for the expression of pol δ by SDS-PAGE (20 μl/*lane*) followed by immunoblotting. *Lanes* are marked according to time of harvest.

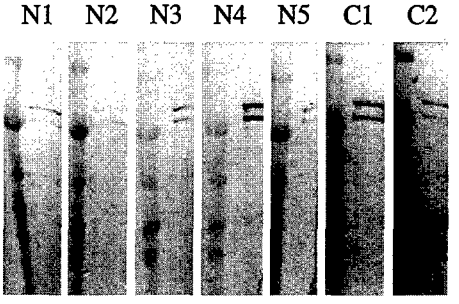

FIG. 3. **Immunoblot of crude recombinant pol δ extract using peptide-specific antibodies.** Sf9 cells were infected with recombinant baculovirus, and the cell extracts were immunoblotted using polyclonal antibodies against specific peptides derived from the NH_2- and COOH-terminal regions of the pol δ sequence (13). These were as follows: N1 (84–101), N2 (129–149), N3 (244–262), N4 (276–295), N5 (312–331), C1 (1047–1068), and C2 (1069–1090). The figure shows a composite of individual blots, each of which shows two *lanes*, the *left lane* being the prestained protein standards and the *right lane*, the Sf9 cell extracts (20 μl, 50 μg of protein).

by immunoblotting with a pol δ monoclonal antibody (38B5; see "Experimental Procedures"). The infected cells were disrupted by passage through a French press in 0.1 M KCl and centrifuged to provide the first extract. The pellet was reextracted by sonication in 0.5 M KCl (second extract). The pellet was then dissolved in 1 ml of 8 M urea. Immunoreactive protein was found to be present in the two salt extracts but not in the urea extract when equal amounts of protein were loaded from each fraction (Fig. 1). These experiments showed that pol δ was expressed as a soluble protein that can be extracted completely by 0.5 M KCl. Immunoblots of the corresponding extracts of Sf9 cells infected with wild type AcMNPV using the same antibody showed the absence of immunoreactive polypeptide (not shown). The time course of pol δ expression was examined by immunoblot analysis of cells taken at intervals after infection with recombinant virus (Fig. 2). For these experiments the 0.1 and 0.5 M KCl extracts were combined. Very little p125 immunoreactivity was observed at 12 h postinfection, and the peak of expression was found to be between 36 and 48 h (Fig. 2).

The recombinant pol δ was immunoblotted using a series of peptide-specific antibodies (Fig. 3) as described by Hao *et al.* (5). The different peptide-specific antibodies (N1, N2, N3, N4, N5, C1, and C2) recognized the recombinant p125 expressed in the baculovirus system. This experiment provided additional confirmation of the identity of the overexpressed protein. Note that the immunoblots (Fig. 3) for p125 appear as a doublet. As we will show, p125 could be purified to a single polypeptide of 125 kDa, although it was often observed as a doublet. A similar behavior was encountered in the isolation of the calf thymus enzyme. At present the most likely explanations are that this may reflect posttranslational modification of the enzyme by phosphorylation or partial proteolysis.

Purification of Recombinant Pol δ—Cells from 80 100-mm plates of Sf9 cells infected with recombinant baculovirus were harvested as described under "Experimental Procedures." A potential complication for the isolation of the recombinant human pol δ from Sf9 is the presence of endogenous DNA polymerases (15), which could compromise studies of the enzymatic properties of human recombinant pol δ. We have circumvented this by passing the crude extract through a phosphocellulose column ("Experimental Procedures"). When the crude extract was chromatographed on a phosphocellulose column, two peaks of activity were detected using poly(dA)·oligo(dT) as a template. One peak eluted at about 0.4 M NaCl and the second at 0.6–0.7 M NaCl (Fig. 4, *center panel*). To determine which of the peaks was the overexpressed pol δ, immunoblots were performed using monoclonal antibody 38B5. Only the first peak of activity (fractions 80–120) was immunoblotted; the second peak (fractions 120–160) did not contain immunoreactive protein (Fig. 4, *top panel*). The second peak also corresponded to the peak of polymerase activity eluted at about 0.7 M KCl when extracts of Sf9 cells infected with wild type AcMNPV baculovirus were chromatographed (Fig. 4, *bottom panel*). DNA polymerase δ isolated from the calf thymus was reported to elute between 235 and 320 mM KCl (3). The second peak was presumed to be endogenous DNA polymerase in baculovirus-infected Sf9 cells, which has been reported to elute from phosphocellulose at high salt concentrations (15).

The peak fractions that immunoblotted with pol δ antibody were pooled, dialyzed, and chromatographed on a Mono Q HPLC column. The column was eluted with a salt gradient as described under "Experimental Procedures" (Fig. 5). Assay of the fractions revealed a peak of DNA polymerase activity which eluted at about 350 mM NaCl. Calf thymus DNA pol δ elutes at 260 mM KCl under the same conditions (3, 4). The preparation contained a 125-kDa polypeptide that was immunoblotted by antibody 38B5 (Fig. 5, *inset*). The recombinant p125 was puri-

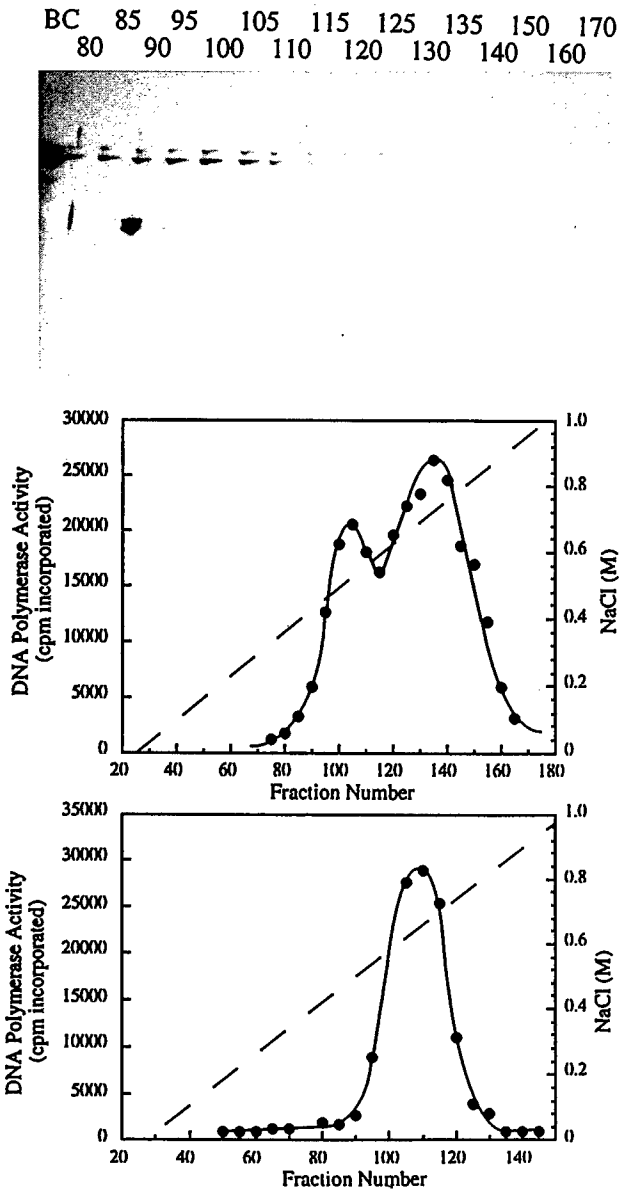

FIG. 4. **Phosphocellulose chromatography of Sf9 cell extracts infected with recombinant baculovirus.** A cell extract from Sf9 cells infected with recombinant baculovirus was chromatographed on phosphocellulose as described under "Experimental Procedures." The fractions were assayed for DNA polymerase activity using poly(dA)·oligo(dT) as template (*center panel*). The fractions containing the two peaks of activity (80–170) were immunoblotted using an antibody against pol δ (38B5) as shown in the *top panel*. *BC* refers to the extract before chromatography. A cell extract from Sf9 cells infected with the control baculovirus was also chromatographed on phosphocellulose, and the fractions were assayed for DNA polymerase activity as shown in the *bottom panel*. Immunoblots of the peak fractions failed to show any immunoreactive protein (not shown).

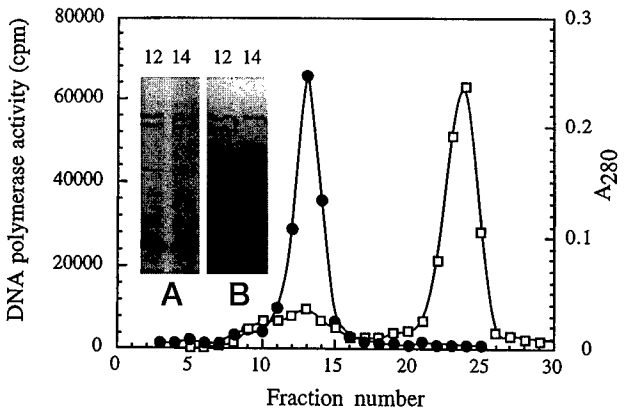

FIG. 5. **Mono Q chromatography of recombinant pol δ.** The peak fractions from the phosphocellulose chromatography step were combined and subjected to HPLC on a Mono Q 5/5 column (see "Experimental Procedures"). The enzyme was eluted with a linear gradient of 0–1 M NaCl in 20 min at 1 ml/min. The fractions were assayed for DNA polymerase activity (*closed circles*). The elution of protein is shown by the absorbance at 280 nm (*squares*). The *inset* shows the SDS-PAGE of fractions 12 and 14, which were stained for protein (*left panel*) and immunoblotted using a monoclonal antibody against pol δ (*right panel*).

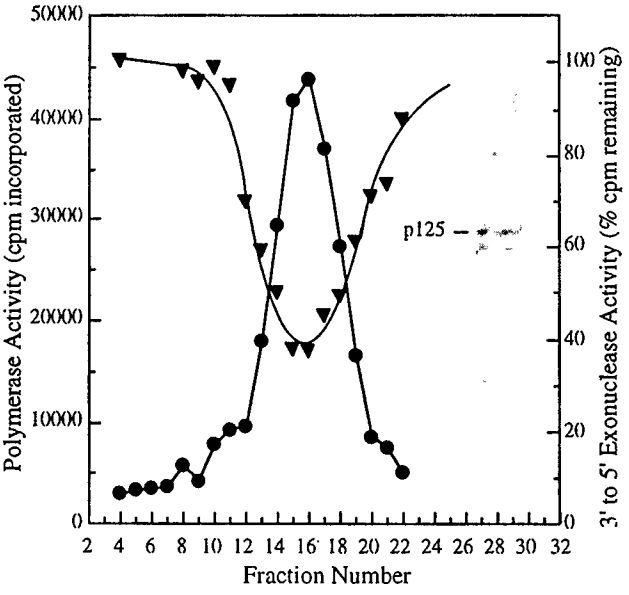

FIG. 6. **Single-stranded DNA-cellulose chromatography.** The fractions from the peak of the Mono Q column which immunoblotted with the pol δ antibody were combined, dialyzed against buffer, and loaded onto a single-stranded DNA-cellulose column as described under "Experimental Procedures." Fractions of 1 ml were collected and assayed for DNA polymerase activity (*circles*) and for exonuclease activity (*inverted triangles*). The *inset* shows the SDS-PAGE of fraction 16, which was stained for protein.

fied to near homogeneity by passage through a single-stranded DNA-cellulose column ("Experimental Procedures"). DNA polymerase activity and exonuclease activities were assayed and found to coelute (Fig. 6). The enzyme was found to be nearly homogeneous as shown by Coomassie Blue staining of SDS-PAGE of the peak fraction (Fig. 6, *inset*).

Immunoaffinity Purification of Recombinant Pol δ—We have shown previously that calf thymus pol δ can be isolated by immunoaffinity chromatography using monoclonal antibody 78F5 coupled to AvidChrom hydrazide (14). Crude Sf9 cell extracts were chromatographed on a pol δ immunoaffinity column ("Experimental Procedures"). The column was washed with buffer containing 50 mM NaCl, and pol δ was eluted by 0.2 M NaCl as shown by analysis for DNA polymerase and exonuclease activities (Fig. 7*A*) and Western blotting (Fig. 7*A*, *inset*). The enzyme obtained was still impure (Fig. 7*A*, *inset*) as determined by SDS-PAGE gels stained for protein. Sf9 cells infected with wild type virus were also passed through this immunoaffinity column, and no detectable DNA polymerase activity was recovered (Fig. 7*A*). This demonstrated that DNA polymerase activities from the Sf9 cells infected with wild type virus did not bind to the column. Note that the overexpressed p125 catalytic subunit could be eluted from the immunoaffinity column by simply using 0.2 M KCl, whereas calf thymus DNA pol δ holoenzyme is eluted at 0.4 M NaCl and 30% ethylene glycol (14). The peak fractions were combined and rechromatographed on the same column. This allowed for the isolation of

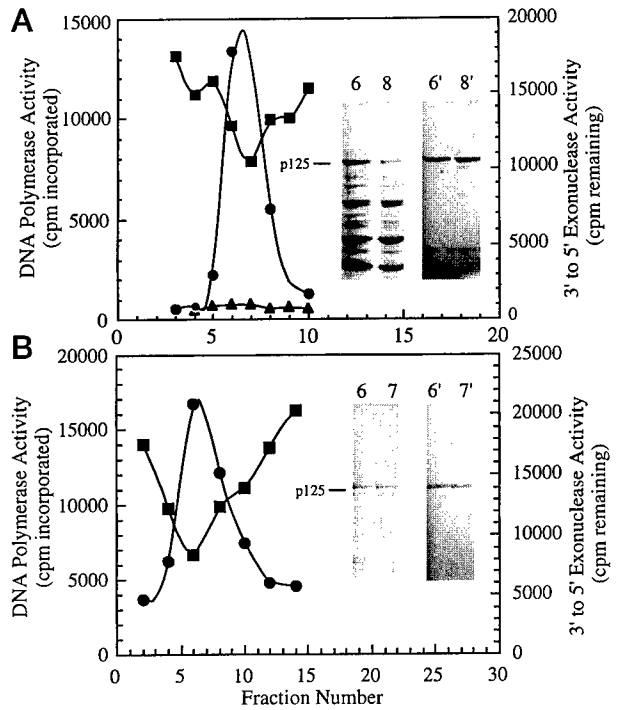

FIG. 7. **Immunoaffinity chromatography of recombinant pol δ.** *Panel A*, an extract from cells infected with recombinant baculovirus was chromatographed on a pol δ immunoaffinity column as described under "Experimental Procedures." The column was eluted with 50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, and 200 mM NaCl. Fractions of 1 ml were collected. The fractions were assayed for DNA polymerase activity (*solid circles*) and for 3′ to 5′ exonuclease activity (*solid squares*). The *inset* shows the SDS-PAGE of fractions 6 and 8 stained for protein with Coomassie Blue. The same fractions were immunoblotted using an antibody against pol δ (*lanes 6′* and *8′*). An extract from cells infected with control baculovirus was also chromatographed on the same column and assayed for DNA polymerase activity (*solid triangles*). *Panel B*, the active fractions from the first immunoaffinity chromatography (*panel A*) were pooled, dialyzed against the equilibration buffer, and rechromatographed on the same column. DNA polymerase and exonuclease activities were assayed as in *panel A*. The *inset* shows the SDS-PAGE of the peak fractions stained for protein and also immunoblotted using an antibody against pol δ.

the recombinant p125 in a nearly homogeneous form (Fig. 7*B*). Starting with 800 mg of total protein in the crude extract, about 0.11 mg of nearly homogeneous protein was recovered, presenting a purification of 153-fold and a final specific activity of 1,200 units/mg of protein using poly(dA)·oligo(dT) as a template (Table I).

Characterization of Recombinant p125—The enzymatic properties of the recombinant pol δ catalytic subunit were compared with those of native calf thymus pol δ, which had been isolated by immunoaffinity chromatography (14), and with the endogenous DNA polymerase activity from Sf9 cells infected with wild type AcMNPV (Fig. 8). The latter was the partially purified preparation obtained after phosphocellulose chromatography (see Fig. 4, *bottom panel*). The activities of the recombinant pol δ catalytic subunit were similar to those of native pol δ and the Sf9 polymerases in that they were inhibited by aphidicolin (Fig. 8*A*) and resistant to 2-(*p*-*n*-butylanilino)-9-(2-deoxy-β-D-ribofuranosyl)adenine 5′-triphosphate (not shown). A well known characteristic of calf thymus pol δ is its sensitivity to inhibition by *N*-ethylmaleimide; recombinant pol δ was inhibited in a manner similar to calf thymus pol δ, whereas the Sf9 polymerase was significantly more resistant to *N*-ethylmaleimide (Fig. 8*B*). The inhibition by low levels of salt is another characteristic of calf thymus pol δ (Fig. 8*C*). Recombinant p125 differed from the calf thymus enzyme in that it was less sensitive to inhibition. The Sf9 DNA polymerase activity was not inhibited but slightly stimulated at 100 mM KCl and was only inhibited at much higher salt concentrations (Fig. 8*C*). The heat inactivation of the three polymerases was also examined. The enzyme was heated to 45 °C and assayed for polymerase activity at the indicated times. DNA polymerase δ from calf thymus and the p125 subunit displayed a similar behavior when heat-treated and were much less sensitive to

TABLE I
Purification of recombinant DNA pol δ p125
Assays was performed using poly(dA) · oligo(dT) template.

Purification step	Protein	Activity	Specific activity	Recovery
	mg	*units*	*units/mg*	%
Cell extract	800	6,272	7.8	100
Phosphocellulose	26	936	36	15
Mono Q HR 5/5	2.2	616	280	20
ssDNA cellulose[a]	0.11	132	1,200	2

ssDNA, single-stranded DNA.

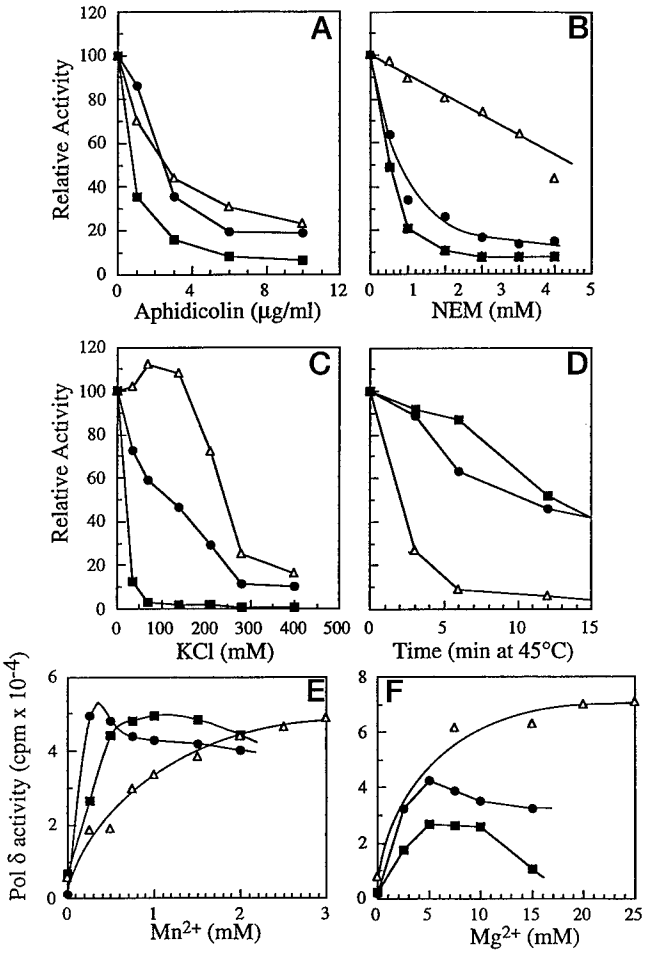

FIG. 8. **Characterization of recombinant pol δ: comparison with native calf thymus pol δ and endogenous DNA polymerases in baculovirus-infected Sf9 cells.** Effects of different compounds and conditions were assayed using poly(dA)·oligo(dT) as a template. Assay conditions were as described under "Experimental Procedures" for the DNA polymerase activities of recombinant pol δ (*closed circles*), native calf thymus pol δ (*closed squares*), and endogenous DNA polymerase from wild type baculovirus overexpressed in Sf9 cells (*open triangles*). PCNA was added in the assays for calf thymus pol δ. The endogenous DNA polymerase from wild type baculovirus overexpressed in Sf9 cells was the material obtained after phosphocellulose chromatography as in Fig. 4, *bottom panel*. *Panel A*, effect of aphidicolin; *panel B*, effect of *N*-ethylmaleimide; *panel C*, effect of KCl; *panel D*, effect of heat treatment at 45 °C for varying amounts of time; *panels E* and *F*, effects of Mn^{2+} and Mg^{2+}, respectively, on the DNA polymerase activity of recombinant pol δ.

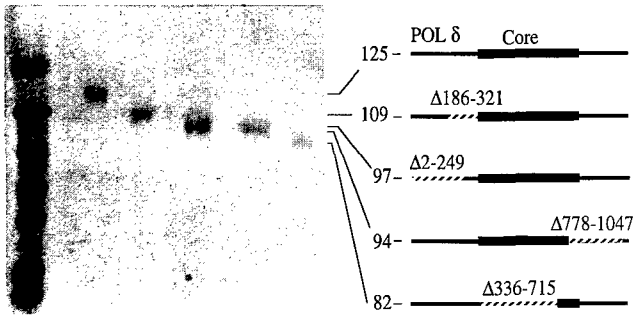

FIG. 9. **Expression of deletion mutants of pol δ p125.** Deletion mutants were constructed as described in Ref. 13. These mutants were purified to homogeneity by phosphocellulose, Mono Q, and single-stranded DNA-cellulose chromatography. The protein staining of the purified mutants after SDS-PAGE are shown. The map of the deletions is shown on the *right*.

heat than the Sf9 polymerase (Fig. 8*D*).

Recombinant pol δ was stimulated by Mn^{2+} in a manner similar to that already known for calf thymus pol δ. Optimal activation was observed between 0.3 and 0.5 mM Mn^{2+}, whereas optimal activity of the Sf9 polymerase was obtained at about 3 mM Mn^{2+} (Fig. 8*E*). Maximal activation of both calf thymus and recombinant pol δ by Mg^{2+} was reached at about 5 mM, whereas the Sf9 polymerase activity was stimulated maximally at 20 mM Mg^{2+} (Fig. 8*F*). These experiments showed that the properties of the recombinant p125 subunit were quite consistent with those of the calf thymus native enzyme.

Deletion Mutagenesis of p125—Extensive compilation and alignment of DNA polymerase sequences from a broad phylogenetic spectrum, *i.e.* from both prokaryotes and eukaryotes, have shown that these fall into two major protein families (16, 17). DNA pol δ belongs to the α-like or B family of DNA polymerases (16). A distinguishing feature of this family is the presence of a conserved core region containing six distinct conserved regions, I–VI, which are thought to contain the catalytic domain for polymerase activity. Unlike pol α, the NH_2-terminal regions of pol δ possess several regions (N1–N5) that are conserved in the Epstein-Barr virus and herpesvirus DNA polymerases (5).

Deletion mutants of the full-length human pol δ (1,107 residues) were constructed. These were p97, in which the N1 and N2 regions of the NH_2 terminus (2–249) were deleted; p109, in which N3, N4, and part of the N5 region including the *Exo*I domain (186–321) were deleted; p82, in which regions IV, A, B, II, VI, and III (336–715) were deleted; and p94, in which regions C, V, CT-1, CT-2, CT-3, and ZnF1 (778–1,047) were deleted (7). These were purified to near homogeneity by phosphocellulose, Mono Q, and single-stranded cellulose chromatography as described above. SDS-PAGE of the mutants (Fig. 9) showed that these had the expected molecular weights. Assays for enzyme activity showed that only p109 (Δ186–321) and p97 (Δ2–249) retained DNA polymerase activity. The p82 and p94 mutants had negligible activities (Table II). This is expected as most of the core region involved in deoxynucleotide interaction was deleted in p82, whereas most of the COOH-terminal domain responsible for DNA interaction was deleted in p94 (Fig. 9).

Evidence for the Phosphorylation of Pol δ by Cyclin-dependent Protein Kinases—Sf9 cells were coinfected with recombinant viruses harboring pol δ and different pairs of recombinant baculoviruses harboring cdk-cyclins. The cdk-cyclin pairs were cdk2-cyclin A, cdk2-cyclin E, cdk4-cyclin D1, cdk4-cyclin D2, cdk4-cyclin D3, cdc2-cyclin A, and cdc2-cyclin B1. After 48 h of infection, the cells were labeled with $^{32}P_i$ for 2 h at 37 °C in low phosphate medium, sonicated, and analyzed by immunoprecipitation using a mixture of pol δ monoclonal antibodies followed by SDS-PAGE and autoradiography as described previously (13). The results (Fig. 10) showed that pol δ was hyperphosphorylated when it was coexpressed with the G_1 phase-specific cdk-cyclins, cdk4-cyclin D3 or cdk2-cyclin E. The relative intensity of phosphorylation when pol δ was coexpressed with these cdk-cyclins was about 10-fold greater than when pol δ was expressed on its own. The relative phosphorylation of pol δ after coinfection with the S or G_2/M-specific cdc2-cyclins (cdc2-cyclin A or cdc2-cyclin B1) was about 20% of that of the G_1/S-specific cdk-cyclins. Cdk2-cyclin A and cdk4-cyclin D2 gave phosphorylation intensities that were similar to the control values obtained when pol δ was expressed alone. The relative intensity of cdk4-cyclin D1 coinfected with pol δ was lower than that of pol δ alone. Our results indicate that pol δ is phosphorylated by cdk4-cyclin D3 and cdk2-cyclin E and is a likely substrate of these G_1/S-specific cdk-cyclins.

TABLE II
Relative specific activities of recombinant p125 and its deletion mutants

Enzymes were purified to near homogeneity as described under "Experimental Procedures" and assayed for DNA polymerase activity using poly(dA) · oligo(dT) as template.

Enzyme	Protein[a]	Specific activity	Relative specific activity
	mg/ml	*units/mg*	
p125	0.0168	1,270	100
Δ186–321	0.0178	1,290	102
Δ2 –249	0.0150	896	71
Δ336–715	0.0195	<0.01	<0.01
Δ778–1047	0.0187	<0.01	<0.01

[a] Concentration of protein in the final preparation was assayed using Coomassie Blue.

Activity of Phosphorylated and Unphosphorylated Forms of Pol δ—The effects of coexpression of p125 with cdk2-cyclin E, cdk2-cyclin A, and cdk4-cyclin D3 on the activity of pol δ were assessed by examination of the activities in the lysates after gel filtration on an HPLC column (Table III). There were no striking effects on the specific activities of the pol δ catalytic subunit assayed using poly(dA)·oligo(dT) as a template (Table III). Immunoblots for the cdk-cyclins in the fractions confirmed that these were also present in the fractions.

Coimmunoprecipitation of Cdk2 and Pol δ—It was found that pol δ could be coimmunoprecipitated with cdk2 from Sf9 cell extracts when they were coexpressed in experiments in which the extracts were immunoprecipitated with antibody against cdk2 and immunoblotted with antibody against pol δ (not shown). The interaction of pol δ with cdk2 was investigated further by examination of the coimmunoprecipitation of deletion mutants of pol δ with cdk2. The results (Fig. 11) showed that all of the deletion mutants tested were coimmunoprecipitated with the exception of the mutant in which the NH_2 terminus (residues 2–249) were deleted. These results demonstrate that there is likely a direct interaction between cdk2 and pol δ, although the possibility that this interaction is mediated by a third protein cannot be discounted.

Coimmunoprecipitation of Pol δ with Members of the Cdk-Cyclins—The coimmunoprecipitation of pol δ with cdk2 could also be observed in cultured Molt 4 cell extracts when cell extracts were immunoprecipitated with pol δ antibody and Western blotted with antibody to cdk2 (Fig. 12, *first lane*). The reciprocal experiment using cdk2 as the precipitating antibody followed by immunoblotting with pol δ antibody also showed that cdk2 was coimmunoprecipitated with pol δ (Fig. 12, *last lane*). When cyclin E was used as the precipitating antibody, the coimmunoprecipitation of pol δ was observed. The coimmunoprecipitation of cdk2 and cdk5 by PCNA antibody was also observed under the same experimental conditions (Fig. 12).

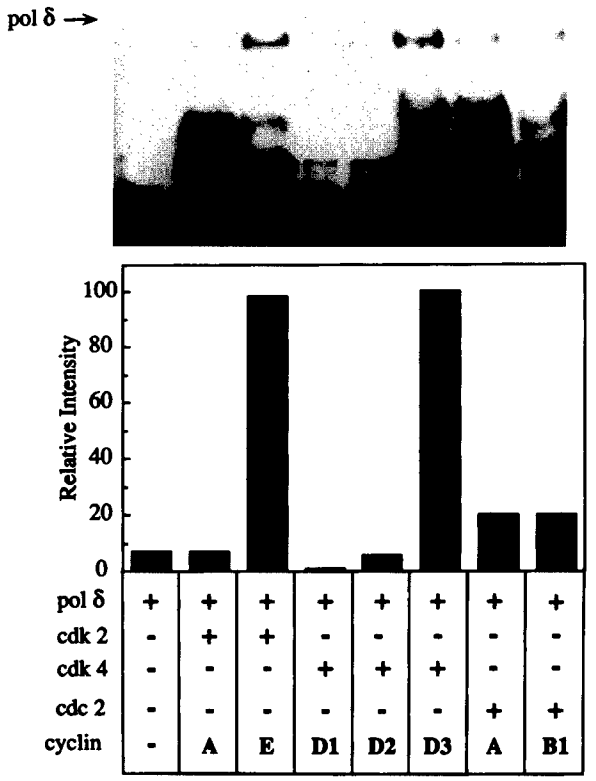

FIG. 10. ***In vivo* phosphorylation of recombinant pol δ in Sf9 insect cells.** The indicated cdk-cyclins and pol δ were coexpressed in Sf9 cells by coinfection as described under "Experimental Procedures." The cells were labeled metabolically with $^{32}P_i$, and the cell lysates were immunoprecipitated with 20 μg of pol δ monoclonal antibody and 40 μl of protein A-Sepharose slurry. The immunoprecipitates were subjected to SDS-PAGE and then autoradiographed (*upper panel*). Relative intensities of the pol δ p125 polypeptide were determined by densitometry.

TABLE III
Specific activities of p125 coinfected with different combinations of cdk-cyclins

Lysates obtained from equal amounts of coinfected cells were precipitated with 50% ammonium sulfate. The precipitates were dissolved in TGEED buffer containing 150 mM KCl, and equal volumes (0.5 ml) of each were loaded onto a Superose 6 HPLC gel filtration column (see "Experimental Procedures"). The results show the protein concentration and pol δ activities of the peak fractions. The presence of the cdk-cyclins in the eluates was confirmed by immunoblot (not shown).

Cotransformant	Protein concentration	Specific activity
	mg/ml	*units/mg*
None	0.33	34
Cdk2	0.36	48
Cdk2-cyclin A	0.36	61
Cdk2-cyclin E	0.79	38
Cdk4-cyclin D3	0.3	34

These experiments show that pol δ interacts with cdk2 and a cyclin *in vivo* and point to the existence of macromolecular complexes between pol δ and the cdk-cyclins.

DISCUSSION

The studies reported here show that the catalytic subunit of DNA pol δ can be expressed in Sf9 cells in an active form and can be isolated by a conventional purification protocol or by an immunoaffinity chromatography procedure. Isolation of the recombinant protein was aided by the use of antibodies against pol δ which did not cross-react with the endogenous DNA polymerase in baculovirus-infected Sf9 cells. We took advantage of an immunoaffinity chromatography procedure to purify

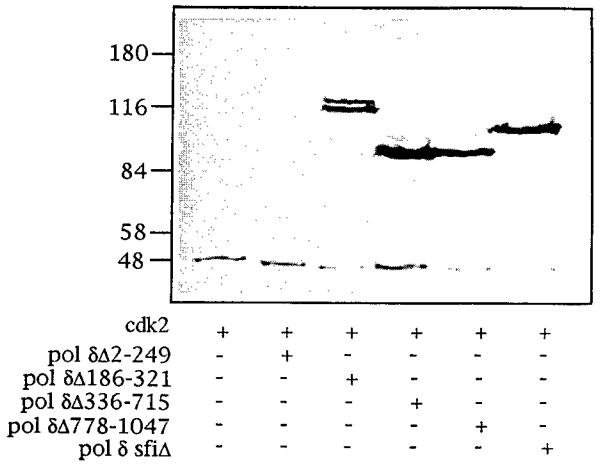

FIG. 11. **Analysis of the ability of the deletion mutants of pol δ to bind to cdk2.** Sf9 cells (about 10^7) were coinfected with pol δ deletion mutants and cdk2 recombinant baculoviruses as indicated. The levels of expression of these mutants were similar as determined by immunoblotting of the Sf9 cell lysates. About 10 mg of total protein from each cell lysate was used for immunoprecipitation with cdk2 polyclonal antibody and SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and immunoblotted with a mixture of NH_2- and COOH-terminal pol δ monoclonal antibodies.

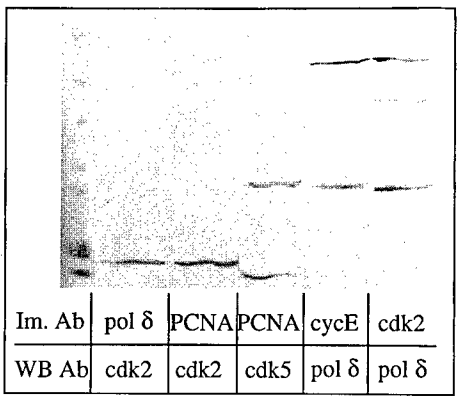

FIG. 12. **Coimmunoprecipitation of pol δ with members of the cdk-cyclin system.** Molt 4 cells were lysed by sonication. About 10 mg of total protein was immunoprecipitated with the first antibody (*Im. Ab*) plus protein A-Sepharose and then Western blotted with a second antibody (*WB Ab*). The *common band* in the *last three lanes* is an artifact (IgG heavy chain).

the recombinant pol δ in a facile manner and to ascertain that it was separated from any endogenous DNA polymerases. The properties of the overexpressed p125 catalytic subunit were compared with those of the native enzyme. Assays of the enzyme activity using poly(dA)·oligo(dT) as a template showed that the specific activities of the preparations were only about 1,200 units/mg (Table I) compared with about 25,000 units/mg protein for the calf thymus holoenzyme (14). This difference is likely the result of the lack of, or of a greatly attenuated response to PCNA by the free catalytic subunit. Other studies of pol δ preparations containing only the catalytic subunit have suggested that it is not PCNA-responsive (18, 19), whereas our previous studies of recombinant pol δ expressed in vaccinia virus have indicated a weak response (2–3-fold stimulation). The baculovirus-expressed pol δ shows little or no response to PCNA, whereas the response is restored by the presence of the p50 subunit (20–22). In other aspects, the enzymatic behavior of the recombinant p125 is very similar to that of the holoenzyme.

Studies of deletion mutants show that deletions (amino acids 2–249 or 186–321) in the NH_2 terminus retain polymerase

activity. Deletions in the core region (amino acids 336–715) and the deletion of regions C and V in the core as well as most of the COOH-terminal region including the zinc finger motifs (778–1047) had no assayable activity (Table II). This is consistent with numerous other studies that indicate that the core region of this family of polymerases is involved in the binding of the incoming dNTP substrate (23, 24) and contains the catalytic center for DNA polymerase activity. The retention of enzymatic activity by the NH_2-terminal deletion mutants is consistent with the existence of a domain structure in which the NH_2-terminal region does not function in catalysis. That this is likely is also consistent with the structure of T4 polymerase, which contains most of the conserved core but only part of the NH_2-terminal region that includes a motif required for the exonuclease activity (5).

The present studies provide the first evidence that the catalytic subunit of pol δ is itself a substrate for cyclin-dependent protein kinases and that this is specific for the G_1 cdk-cyclins because other cdk-cyclin combinations were less effective in phosphorylating pol δ when they were coexpressed in Sf9 insect cells. Although the *in vivo* kinase activity of cdk-cyclin overexpressed in Sf9 insect cells may not reflect actual cellular events in the mammalian cell cycle, the involvement of G_1 phase cdk-cyclins is consistent with our previous observations that pol δ is phosphorylated *in vivo* during the cell cycle and is maximal near the G_1/S transition (25). The primary structure of pol δ shows a number of potential phosphorylation sites for the cdks, including six sites possessing the (S/T)P motif: serines 207 and 788 and threonines 83, 150, 238, and 640 (25). It is well known that in mammalian cells the key regulators of the transition from G_1 to S phase of the cell cycle include the G_1 cyclins-three D type cyclins (D1, D2, D3) and cyclin E (26). Cyclin E expression is periodic, peaks at the G_1/S transition, and regulates S phase commitment together with its catalytic subunit cdk2. Unlike cyclin E, expression of D type cyclins is cell lineage-specific and highly mitogen-dependent, rising on growth factor stimulation and declining rapidly on growth factor withdrawal (27, 28). The current model for G_1 cdk-cyclin functions is that cyclin D binds directly to the tumor suppressor gene product pRb, targeting cdk4 to its substrate, and resulting in phosphorylation of pRb during middle to late G_1 phase. This reverses the growth-suppressive effects of pRb by releasing transcriptional factor E2F from its inhibitory constraint; the untethered E2F factor is then able to activate a series of genes required for DNA replication (26). The G_1 cdk-cyclins are also thought to phosphorylate other key substrates resident at the DNA replication origin to trigger the actual onset of DNA replication once cells pass the restriction point (29, 30). Pol δ is the central enzyme in eukaryotic DNA replication and is tethered to DNA by a direct interaction with the PCNA clamp, which converts pol δ from a distributive into a highly processive enzyme for DNA synthesis (31, 32). Thus, the finding that pol δ is a substrate for the G_1 cdk-cyclins is of significance as it provides a potential linkage for the cell cycle control of DNA synthesis. However, our studies do not reveal any major effects of phosphorylation on the activity of the p125 catalytic subunit, and only small increases (<2-fold) were observed after coexpression with cdk-cyclins (Table III). Pol α-primase has also been shown to be phosphorylated, and phosphorylation does not or only moderately changes its enzymatic properties (33–35). However, the ability of pol α-primase to initiate SV40 DNA replication *in vitro* was found to be inhibited markedly after phosphorylation by cyclin A-dependent kinases (36).

Examination of the interaction of cdk2 with the deletion mutants of pol δ showed that the tertiary structure of pol δ is not required for this interaction and that the binding region is located in the NH_2-terminal 249 residues of pol δ. The NH_2-terminus of yeast and mammalian pol δ harbors several highly conserved regions (N1–N5) that are also present in herpes and Epstein-Barr viral polymerases (5). These conserved regions are likely protein-protein interaction sites for pol δ (5). The binding site of pol δ for PCNA has been mapped to the N2 region (13). The data presented also provide the first evidence for complexes that involve pol δ and the cdk-cyclins. The targeting of the cdks to a substrate has some precedence since the G_1 cdk-cyclins are known to form complexes with pRb. The obvious question is whether this has any functional physiological significance in relation to the phosphorylation or regulation of pol δ. The present findings show that the interaction of pol δ with cdk2 and cdk4 needs to be investigated further, in addition to the issue of the cellular role of phosphorylation of pol δ by the cdk-cyclins.

There are many levels at which phosphorylation could affect pol δ function other than the simple modulation of enzyme activity in a simple assay. This is apparent because physiologically pol δ is part of a holoenzyme and part of an extended multiprotein complex. Current findings that p21, a potent inhibitor of G_1 cdks, and pol δ compete for the same sites in the interdomain connector loop of PCNA (37, 38) add even more complexity to these questions. Xiong *et al.* (39, 40) observed that PCNA is in a quaternary complex that includes cyclin D, cyclin-dependent kinases (cdk2, cdk4, cdk5), and p21. No phosphorylation of PCNA and p21 was detected, suggesting that neither of them is the primary substrate of phosphorylation. Thus, there are many possible permutations and speculations possible as to how regulatory systems could emerge from this melange of potential complexes. We have obtained preliminary evidence that pol δ is a substrate for the cyclin-dependent protein kinases. This was shown by the coexpression of baculovirus vectors for pol δ with several different cdk-cyclin combinations in Sf9 cells (Fig. 10) and coimmunoprecipitation Western blot studies in Molt 4 cells (Fig. 12). These results suggest that more than one cyclin might regulate pol δ, possibly triggering its phosphorylation at different sites or times of the cell cycle. Coimmunoprecipitation of pol δ deletion mutants with cdk2 also established the site of interaction (Fig. 11). Although the regulation of pol δ by protein phosphorylation has yet to be demonstrated firmly, this possibility provides a potential mechanism that might provide for the temporal regulation of DNA synthesis in concert with the cell cycle.

Although the present evidence indicates that the phosphorylation status of the catalytic subunit of DNA polymerase δ may have no significant effect on its activity, the question of whether phosphorylation has any physiological relevance in affecting or regulating the biological function of polymerase δ still needs to be answered. A role of phosphorylation or binding of the kinase in affecting the properties of the polymerase *in vivo* in modulating the function of pol δ in DNA replication or repair cannot be excluded. In this regard, note that significant difference was observed when replication protein A is phosphorylated in SV40 DNA replication (41–43) and nucleotide excision repair systems (42). Further studies are needed to answer the question of the regulatory consequences of phosphorylation of pol δ and for that matter other replication proteins. The putative kinase consensus sequences in pol δ also show that it could be a substrate for DNA-dependent protein kinase. The latter kinase phosphorylates serine or threonine residues that are followed or preceded by glutamine residues (S/T)-Q or Q-(S/T). It remains to be determined whether other kinases, *e.g.* DNA-dependent protein kinase, are also involved in the phosphorylation of the catalytic subunit of pol δ.

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Direct Interaction of Proliferating Cell Nuclear Antigen with the p125 Catalytic Subunit of Mammalian DNA Polymerase δ*

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The formation of a complex between DNA polymerase δ (pol δ) and its sliding clamp, proliferating cell nuclear antigen (PCNA), is responsible for the maintenance of processive DNA synthesis at the leading strand of the replication fork. In this study, the ability of the p125 catalytic subunit of DNA polymerase δ to engage in protein-protein interactions with PCNA was established by biochemical and genetic methods. p125 and PCNA were shown to co-immunoprecipitate from either calf thymus or HeLa extracts, or when they were ectopically co-expressed in Cos 7 cells. Because pol δ is a multimeric protein, this interaction could be indirect. Thus, rigorous evidence was sought for a direct interaction of the p125 catalytic subunit and PCNA. To do this, the ability of recombinant p125 to interact with PCNA was established by biochemical means. p125 co-expressed with PCNA in Sf9 cells was shown to form a physical complex that can be detected on gel filtration and that can be cross-linked with the bifunctional cross-linking agent sulfo-ethylene glycolbis (sulfosuccinimidyl succinate). An interaction between p125 and PCNA could also be demonstrated in the yeast two hybrid system. Overlay experiments using biotinylated PCNA showed that the free p125 subunit interacts with PCNA. The PCNA overlay blotting method was also used to demonstrate the binding of synthetic peptides corresponding to the N2 region of pol δ and provides evidence for a site on pol δ that is involved in the protein-protein interactions between PCNA and pol δ. This region contains a sequence that is a potential member of the PCNA binding motif found in other PCNA-binding proteins. These studies provide an unequivocal demonstration that the p125 subunit of pol δ interacts with PCNA.

Proliferating cell nuclear antigen (PCNA)[1] was originally discovered as an antigen in autoimmune sera from patients with systemic lupus erythematosus and was reported to be found only in actively proliferating cells (1). It was later shown to be a factor that enhanced the processivity of DNA polymerase δ (pol δ) and to have key roles in both DNA replication and repair (2, 3). There have been striking recent advances in our understanding of the structure and functions of PCNA (4). Purification and expression of human recombinant PCNA and its physiochemical characterization established that it was a trimeric protein (5). The crystal structures of both yeast and human PCNA have been determined (6, 7). Like the T4 gene 45 protein and the β subunit of *Escherichia coli* DNA polymerase III holoenzyme, PCNA functions as a sliding DNA clamp that forms a closed ring around duplex DNA (8). The binding of pol δ to PCNA provides an elegant micromechanical solution to the biological need to maintain an extraordinarily high level of processivity during the synthesis of chromosomal DNA (8–10). Recently, it has also been found that PCNA has a number of protein partners with which it interacts (4, 9, 11). Pol δ has been shown to be involved not only in DNA replication but also in DNA repair and can be regulated by cell cycle proteins (2, 12, 13). Thus, an important area of interest is the protein-protein interaction sites of PCNA, because it may be the nexus for multiple protein-protein interactions involved in replication, repair, recombination, and cell cycle regulation.

The pol δ core enzyme consists of two subunits, p125 and p50 (14). Previous work from this laboratory has implicated the p125 subunit in an interaction with PCNA (10). A synthetic peptide conforming to the N2 region (residues 129–149) was found to inhibit PCNA stimulation of pol δ isolated from calf thymus (10). p125 and PCNA co-expressed in Sf9 cells could be co-immunoprecipitated with an antibody to PCNA, showing that the catalytic subunit of DNA polymerase δ interacted with PCNA (10). However, the recombinant p125 catalytic subunit can only be stimulated by PCNA at most 2–3-fold, and the presence of the p50 subunit is required to restore a significant level of PCNA stimulation of the p125 subunit (15–17).

Two recent studies of yeast pol δ reported contrary results. No evidence for a direct interaction between the *Schizosaccharomyces pombe* pol δ p125 subunit and PCNA could be found, either by co-immunoprecipitation experiments after their co-expression in insect cells or by a yeast two hybrid assay (18). It was concluded that no direct interaction occurs between *S. pombe* p125 and PCNA. In *Saccharomyces cerevisiae*, similar results were obtained using a PCNA overlay assay. In the latter studies, the interaction of *S. cerevisiae* p125 and p58, as well as the recently identified third subunit (p55), was studied by a PCNA overlay method. It was shown that only the third subunit (p55) of pol δ interacted with PCNA (19), indicating that the interaction of pol δ with PCNA involved the third subunit, whereas no evidence could be obtained for an interaction of PCNA with either the small second subunit p58 or the catalytic subunit.

These findings are in conflict with our previous studies of the p125-PCNA interaction, and they suggest that the binding of PCNA in yeast and human depend on different subunit-PCNA interactions. Alternatively, it is also possible that the interaction of PCNA with pol δ involves multiple interactions with pol δ subunits. For this reason, we have undertaken a rigorous

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[1] The abbreviations used are: PCNA, proliferating cell nuclear antigen; pol δ, polymerase δ; EGS, ethylene glycolbis(sulfosuccinimidyl succinate); PAGE, polyacrylamide gel electrophoresis.

examination of the protein-protein interactions of the p125 subunit of pol δ and PCNA using different biochemical methods. Our studies leave little doubt of the ability of the p125 subunit to interact with PCNA.

EXPERIMENTAL PROCEDURES

Ectopic Expression of p125 and PCNA in Cos-7 Cells—COS-7 cells were cultured in Dulbecco's medium (Life Technologies, Inc.) containing 10% fetal bovine serum. These cells were transfected with c-Myc-His-PCNA and pCMV-p125 using the calcium phosphate method. For each 9-cm Petri dish, the transfected DNA consisted of 5 μg of each expression vector and 10 μg of Bluescript SK DNA. The cells were washed with phosphate-buffered saline and scraped in 1 ml of radioimmune precipitation buffer (10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride). After 15 min on ice, the lysed cells were spun at 5000 rpm for 15 min at 4 °C. Immunoprecipitations were performed using anti-Myc antibody (Roche Molecular Biochemicals) in 1 ml of extract. The immunoprecipitates were separated on a 5–15% gradient SDS gel and immunoblotted with anti-His antibody (Invitrogen) or pol δ monoclonal antibody. The blot was then detected by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Gel Filtration of Sf9 Lysates Expressing Recombinant p125 and PCNA—Sf9 cells were grown in 75-cm^2 flasks to 75% confluence. The cells were dislodged from 25 flasks by pipetting 5 ml of fresh medium into the flasks. Cell pellets were collected by centrifugation at 3000 × *g* for 10 min. Recombinant baculoviruses containing the human p125 coding sequence, its deletion mutants, or PCNA were as described previously (13). Sf9 cells were infected with either the p125 or the deleted p125 and PCNA at a multiplicity of infection of 5 for each recombinant baculovirus. The cells were incubated in the viral inoculum for 2 h with gentle rotation every 15 min and then centrifuged at 3000 × *g* for another 10 min. The inoculum was removed, and cell pellets were suspended in 150 ml of fresh medium divided into 2 flasks and incubated for another 60 h.

The cell lysates from a total of 2 × 10^8 cells were collected and processed as described previously (13). One mg of protein in a total volume of 0.5 ml was chromatographed on a Sephacryl S-300 column (1.5 × 100 cm). Fractions of 1.5 ml were collected. The elution of pol δ activity was monitored by its activity on poly(dA)/oligo(dT) as described previously (10)

Cross-linking of p125 and PCNA Expressed in Sf9 Cells—The peak fraction of the PCNA-p125 complex from the S-300 chromatography was then subjected to cross-linking with sulfo-ethylene glycolbis (sulfosuccinimidyl succinate) (sulfo-EGS) at the indicated concentrations for 15 min at room temperature as described previously (5). The cross-linked species were identified by Western blotting. Western blot was performed using 78F5 and 38B5 pol δ monoclonal antibodies (13). Prestained protein standards (Sigma) were used as molecular weight markers and also to provide for visual confirmation of efficient transfer. Nitrocellulose blots were blocked in 5% (w/v) nonfat dry milk in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20 (TBST) for 1 h at room temperature. The blot was then incubated with monoclonal antibodies to pol δ for 12 h. at 25 °C. After three 10-min washes in TBST, the blot was incubated with anti-mouse IgG-horseradish peroxide conjugate diluted in TBST (1:10,000) for 1 h at room temperature with constant rocking. The blot was then washed five times with TBST for 20 min each and developed by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Overlay Blotting with Biotinylated PCNA—Recombinant PCNA was expressed in *E. coli* and purified to near homogeneity as described previously (5). PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (Amersham Pharmacia Biotech). The reaction mixtures contained 0.5 mg of PCNA, 0.5 mg/ml biotinamidocaproate *N*-hydroxysuccinamide ester in a total volume of 0.5 ml in 20 mM bicarbonate buffer, pH 8.6. After reaction for 60 min at room temperature, the PCNA was purified on a G25 Sephadex column (5-ml bed volume) equilibrated in phosphate buffered saline containing 1% bovine serum albumin. Samples containing 1–5 μg of protein were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Prestained protein standards (New England Biolabs) were used as molecular weight markers. The nitrocellulose membranes were blocked with 5% nonfat dry milk in TBST for 45 min at room temperature followed by three washes of TBST for 10 min each. The blots were then incubated with biotinylated PCNA (1 μg/μl) diluted in TBST (1:900) at 4 °C overnight. The blots were washed five times with TBST for 15 min followed by incubation with streptavidin-horseradish peroxidase conjugate diluted in TBST (1:5000) for 1 h at room temperature with constant rocking. The blots were then washed five times with TBST for 20 min each and developed using by chemiluminescence (ECL detection system, Amersham Pharmacia Biotech).

Dot Blot Analysis of Binding of PCNA to Synthetic Peptides—Peptides to p21 and the N2 region of pol δ p125 were synthesized by Bio-Synthesis Co. The peptides were dissolved in water at a concentration of 5 μg/μl. Five μl of each peptide were dot blotted onto nitrocellulose membranes. Bovine serum albumin, PCNA, p125 and purified calf thymus pol δ (25 μg) were dot blotted onto the same membranes. The nitrocellulose membranes were then blocked and blotted with biotinylated PCNA as described above.

Pairwise Yeast Two Hybrid Interactions—Plasmids expressing the GAL4 fusions with the coding sequences for p125, p50 and PCNA were constructed in the pAS2–1 and pACT2 vectors (CLONTECH Laboratories Inc.). Sequential transformations of Y190 were performed by the lithium acetate method with dimethyl sulfoxide at a final concentration of 10%. pAS2–1-p125 and pAS2-p50 were transformed into Y190 and plated onto Trp^- plates. Y190 stains transformed with pAS2–1-p125 or pAS2–1-p50 were then transformed with pACT2–50 or pACT2-PCNA. After overnight recovery in Trp^-Leu^- medium, the transformants were plated on Trp^-Leu^- His^-/3-AT plates to select for histidine prototrophy. For the liquid assay, GAL4 and T antigen/p53 were transformed into Y190 as positive controls. pAS2–1-p50 and pAS2–1-PCNA in Y190 were used as negative controls. One ml of overnight yeast culture in liquid $Trp^-Leu^-His^-$/SD selection medium was prepared. Four ml of YPD medium (yeast culture medium containing 20 g/liter Difco peptone, 10 g/liter yeast extract, and 2% glucose) was added to the overnight culture and incubated for 5 h at room temperature with shaking (230–250 rpm). The A_{600} of the culture was recorded. One ml of cells was centrifuged at 1,000 × *g* for 30 s and washed once with 1 ml of Z buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgCl_2{\cdot}7H_2O$) followed by another centrifugation. The cell pellet was resuspended in 100 μl of Z buffer. The cells were disrupted by freeze-thaw (liquid nitrogen for 1 min, thawed at 30 °C for 1 min). Z buffer (0.7 ml) and β-mercaptoethanol at a final concentration of 40 mM was added. *O*-Nitrophenyl β-D-galactopyranoside (160 μl, 2.2 mM in Z buffer) was added, and the reaction was incubated at 30 °C for 3–90 min. (Positive strong interactions were incubated for 3 min and negative interactions for 90 min.) The reactions were terminated by addition of 0.4 ml of 1 M Na_2CO_3. Cell debris was removed by centrifugation at 10,000 × *g* for 2 min, and the A_{420} was recorded. The β-galactosidase activity was calculated. Arbitrary units of activity were calculated as: β-galactosidase units = $1000 \times A_{420}/(t \times V \times A_{600})$, where t = min of incubation; V = 0.1 ml.

RESULTS

Co-immunoprecipitation of p125 and PCNA from Crude Calf Thymus and HeLa Extracts and after Their Ectopic Expression in Cultured Cells—Previous studies have demonstrated that human PCNA could be co-immunoprecipitated with the p125 catalytic subunit of pol δ from Sf9 insect cell lysates, under conditions where both proteins were overexpressed as recombinant proteins (10). It can be argued that the interactions observed were a consequence of the supranormal concentrations of both proteins under these conditions of overexpression and may not reflect the behavior of the two proteins in a normal cellular context. Studies were therefore performed to establish whether PCNA and p125 could be co-immunoprecipitated from mammalian tissue or cell culture extracts. The results show that p125 and PCNA can be readily co-immunoprecipitated from crude calf thymus extracts that had been partially purified on phenyl agarose, as well as from HeLa cell lysates (Fig. 1, *A* and *B*). Because of the multisubunit nature of pol δ, these experiments do not show a direct interaction between p125 and PCNA, but they do confirm that an interaction between pol δ and PCNA is readily demonstrated in cell extracts. F1

Next, evidence for an interaction between p125 and PCNA when they are ectopically expressed in mammalian cultured cells was sought. Human PCNA was expressed in Cos 7 cells with dual tags (a hexahistidine tag and a c-Myc tag) together with p125 expressed in the pCMV vector (see under "Experimental Procedures"). Samples of the cell extracts were immu-

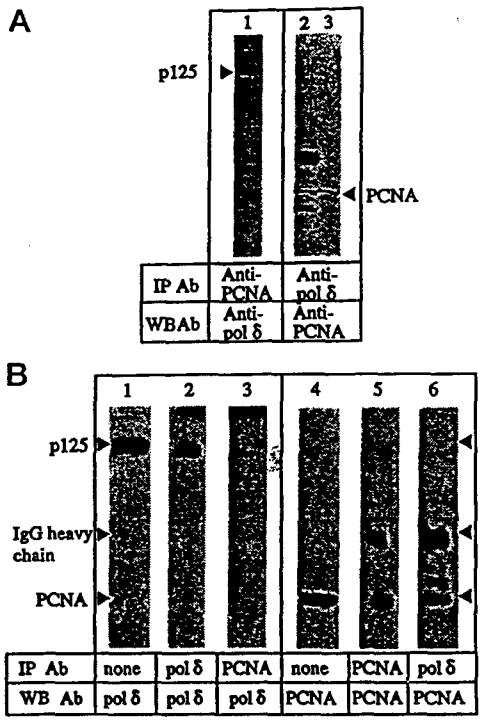


FIG. 1. **Co-immunoprecipitation of p125 and PCNA with monoclonal antibodies from calf thymus extracts or HeLa extracts.** *A, lane 1,* a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated (*IP*) with 20 μg of 74B1 PCNA monoclonal antibody and blotted with monoclonal antibody 78F5 against pol δ. *Lane 2,* a calf thymus extract was passed through phenyl agarose, and 50 μl of the extract was immunoprecipitated with 78F5 pol δ antibody and immunoblotted with 20 μg of 74B1 monoclonal antibody against PCNA. *Lane 3,* control containing purified PCNA, immunoblotted with the antibody against PCNA. Western blotting (*WB*) was performed using biotinylated sheep anti-mouse immunoglobulin as second antibody followed by incubation with streptavidin-biotinylated horseradish peroxidase complex. Color development was performed by incubation with 4-chloro-1-naphthol and hydrogen peroxide and terminated with sodium azide. *B,* HeLa cells were lysed in 1% Nonidet P-40 lysis buffer and protease inhibitors. The lysate was precleared with protein G agarose bead before incubation with antibodies (*Ab*). HeLa lysate (2 mg of protein) was incubated with 30 μg of monoclonal antibody for each immunoprecipitation. Immunobeads were suspended in 150 μl of lysis buffer and 150 μl of 2× SDS loading buffer. *Lanes 1* and *4,* 50 μg of untreated crude HeLa lysate. *Lanes 2* and *6,* 50 μl of immunoprecipitate from monoclonal 38B5 against pol δ. *Lanes 3* and *5,* 50 μl of immunoprecipitate from monoclonal antibody 74B1 against PCNA. Samples were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. The membrane was divided into two halves. *Lanes 1–3* were immunoblotted with monoclonal antibody 78F5 against pol δ. *Lanes 4–6* were immunoblotted with monoclonal antibody 74B1 against PCNA. Western blotting was performed using horseradish peroxidase anti-mouse immunoglobulin as a second antibody followed by chemiluminescence detection.

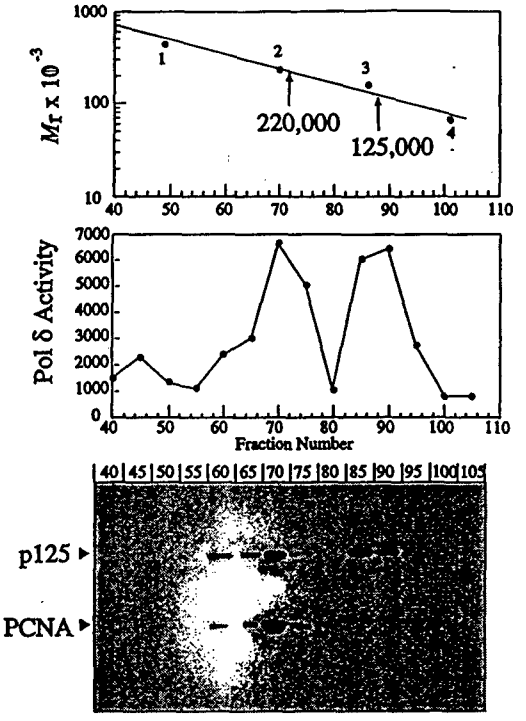


FIG. 2. **Gel filtration of Sf9 lysates expressing recombinant p125 and PCNA.** p125 and PCNA were co-expressed in Sf9 cells, and the cell lysates (0.5 ml, 1 mg of protein) were chromatographed on Sephacryl-S-300 (see under "Experimental Procedures"). The elution of pol δ activity was monitored by activity assay (*center panel*) and for p125 and PCNA by Western blotting using a mixture of monoclonal antibodies to p125 and PCNA (*bottom panel*). The column was calibrated using ferritin (molecular weight 440,000), catalase (232,000), aldolase (158,000), and bovine serum albumin (67,000), shown as *1–4* in the *upper panel*. The approximate molecular weights of the two peaks of pol δ activity were 220,000 and 125,000, as shown by the *arrows* in the *upper panel*.

noprecipitated using a c-Myc antibody and were Western blotted with pol δ antibody. The results show that p125 is co-immunoprecipitated with c-Myc-His-tagged PCNA (not shown). Control experiments in which the immunoprecipitates were blotted with anti-His antibody confirmed that PCNA was present in the immunoprecipitate. These experiments demonstrate that pol δ p125 can be shown to co-immunoprecipitate with PCNA, either when endogenous pol δ is present or when p125 is ectopically co-expressed with PCNA.

Formation of a Physical Complex between p125 and PCNA—The experimental results in which p125 co-expressed with PCNA in either baculovirus (10) or in mammalian cells (Fig. 1) indicated that p125 is able to interact with PCNA and imply that this interaction is independent of the presence of p50 or other subunits of pol δ. In these experiments, co-immunoprecipitation does not provide rigorous evidence that the p125 subunit directly interacts with PCNA, as it is possible that the p125 that is detected by co-immunoprecipitation is by virtue of an interaction of PCNA with a pol δ heterodimer, *i.e.*, the interactions are mediated by a third polypeptide(s). In order to provide a more rigorous test, Sf9 cells were co-infected with baculovirus vectors for p125 and for PCNA. The Sf9 cell lysates were then subjected to gel filtration on a Sephacryl S-300 column (Fig. 2). Assays for pol δ activity showed that there were two peaks of activity, one with a relative molecular weight of 125,000 and the second with a relative molecular weight of 220,000 (Fig. 2, *upper* and *middle panels*). The fractions were analyzed for the presence of p125 and PCNA by Western blotting using specific antibodies. The results show that the 125,000 peak contained only p125, whereas PCNA co-migrated with p125 in the 220,00 molecular weight fractions. Furthermore, no free PCNA was detected in the range where either the dimer or trimer form would be expected to migrate. (In previous studies of the behavior of recombinant PCNA expressed in *E. coli,* we had shown that PCNA in solution is a mixture of dimers and trimers (5)). These results provide the first direct demonstration that free p125 forms a physical complex with PCNA, and moreover, the apparent molecular weight is consistent with a complex of p125 with a trimeric form of PCNA, because previous work showed that the PCNA trimer migrates with an apparent molecular weight of approximately 100,000 on gel filtration (5).

In order to establish that the co-elution of p125 and PCNA is not due to fortuitous associations with unrelated proteins, cross-linking experiments using bifunctional cross-linking

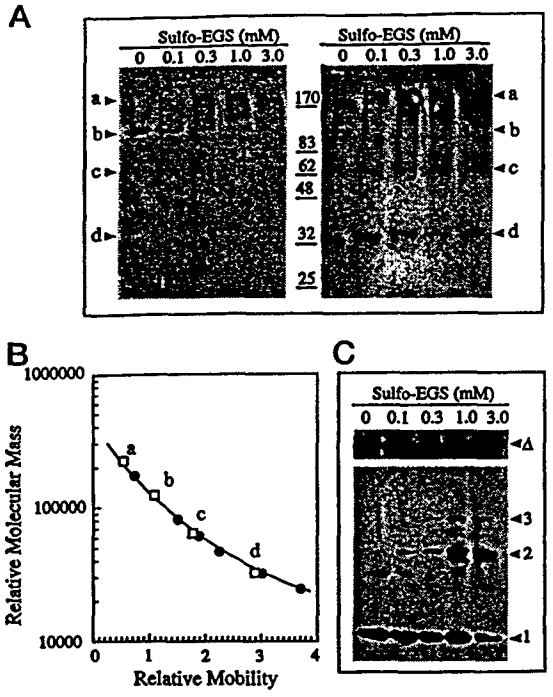


FIG. 3. Cross-linking analysis of the p125/PCNA complex. Samples of the peak fraction number 66, (40 μl, 20 ng of protein) from the S-300 chromatography (Fig. 3) were incubated with sulfo-EGS at the indicated concentrations (mM) for 15 min at room temperature and then subjected to SDS-PAGE followed by Western blotting using a monoclonal antibody against p125 (*A, left panel*) and a monoclonal antibody against PCNA (*A, right panel*). *Arrows a* and *b* show p125 immunoreactive bands of 225 and 125 kDa, respectively; *arrow c* corresponds to the cross-linked PCNA dimer, and *arrow d* corresponds to the PCNA monomer. *B* shows the determination of the relative molecular masses of the *bands a–d* (*open squares*); prestained protein standards (New England Biolabs. Inc.) were used as markers (*solid circles*). *C*, a similar cross-linking experiment was performed using a deletion mutant of p125 (Δ2–249) in which the N-terminal 249 residues were deleted (13). The *upper panel* shows the Western blot of the 97 kDa immunoreactive p125 (Δ2–249) (Δ), which was not depleted during the course of the experiment. In addition, no significant formation of higher molecular weight species was observed (not shown). The *lower panel* shows the immunoblot with antibody against PCNA, where *1–3* show the positions of the monomer, dimer, and trimer forms of PCNA, respectively, without evidence for formation of cross-links with p125 (Δ2–249).

agents were performed to establish a direct p125-PCNA protein-protein interaction in the 220,000 molecular weight complex. Sulfo-EGS was used, as we have previously shown that EGS readily cross-links PCNA (5). When the peak fractions of the complex of p125 and PCNA obtained on gel filtration were cross-linked with sulfo-EGS, and the formation of cross-linked species monitored by Western blotting with antibodies against p125 (Fig. 3*A, left panel*) or PCNA (Fig. 3*A, right panel*), a rapid disappearance of the PCNA monomer and p125 was observed with the concomitant formation of a band of 225 kDa that reacted with both p125 and PCNA antibodies. This is consistent with the presence of p125 in complex with a PCNA trimer. It is noteworthy that no accumulation of species that might represent p125 cross-linked to a PCNA monomer or a PCNA dimer was observed.

Control cross-linking experiments with Sf9 cell lysates expressing PCNA or p125 alone were performed. In the case of p125, no cross-linking with sulfo-EGS was observed under the conditions used (not shown). In the case of PCNA, sulfo-EGS gave exactly the same pattern of cross-linking that we had previously found for PCNA cross-linked with EGS (5), in that the major species of PCNA that were observed after cross-linking were the monomer, the dimer, and a smaller amount of the trimer.

In a parallel experiment, a deletion mutant of p125 (Δ2–249) in which the N-terminal 248 residues were removed (13) was co-expressed with PCNA and subjected to cross-linking. In this case, only the monomer, dimer, and trimer species of PCNA were observed, as for PCNA alone, as shown in Fig. 3*C*. Blotting with pol δ antibodies also showed that there were no cross-links formed with the N-terminal deletion mutant of pol δ (Fig. 3*C, upper panel*). These results are consistent with our previous observations that the N-terminal region of p125 is required for its interaction with PCNA.

Far Western Blotting with PCNA—In order to obtain additional evidence that p125 can interact with PCNA, an overlay technique for blotting of PCNA-binding proteins was used. PCNA was labeled with biotin as described under "Experimental Procedures." Pol δ was subjected to SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were then blotted with biotinylated PCNA (see under "Experimental Procedures") and visualized using a chemiluminescence method. A number of experiments were performed; they showed that only the p125 band provided a strong reaction with biotinylated PCNA. When immunoaffinity purified calf thymus pol δ enzyme was blotted with biotinylated PCNA, a strong reaction was seen with the p125 catalytic subunit (Fig. 4*A, left panel*). The p50 subunit of pol δ did not interact with PCNA in the overlay experiments (Fig. 4*A, left panel*). The inability of the p50 subunit to interact with PCNA was confirmed using purified recombinant p50 subunit (Fig. 4*A, left panel*). This experiment was repeated using recombinant pol δ heterodimer produced by overexpression of p125 and p50 in Sf9 cells. The same results were obtained, namely that p125 but not p50 reacted with biotinylated PCNA (Fig. 4*B*). Because the overlay depends on an interaction with a polypeptide band separated by SDS-PAGE, this positive interaction demonstrates that the p125 subunit interacts with PCNA in a manner that is independent of the presence of the other subunits of pol δ. In Fig. 4*A*, it is noted that the overlay of immunoaffinity purified pol δ with PCNA reveals a doublet of 70 kDa. In other studies, partial protein sequence was obtained of this band, and a BLAST search identified this polypeptide as KIAA0039 (GenBank™). This was found to be a mammalian counterpart of *S. pombe* Cdc27.[2] F4 Fn2

We have also examined the Δ2–249 N-terminal deletion mutant of p125, in which the N2 region is absent (13), by PCNA overlay. The results (Fig. 5*A*) show that this deletion mutant is not recognized by overlay with biotinylated PCNA. In parallel, experiments were performed using deletion mutants Δ186–321, Δ336–715 (core region deleted), Δ675–1107, and Δ778–1047 (C-terminal regions deleted) expressed in Sf9 cells (Fig. 5*B*). All of these deletion mutants, covering essentially the entire p125 sequence from residue 186 (Fig. 5*C*), interacted with PCNA. These results, taken with the inability of the Δ2–249 deletion mutant to bind to biotinylated PCNA, restricts the binding region on p125 to within the first 186 residues. F5

Yeast Two Hybrid Assay—Human p125, p50 and PCNA coding sequences were inserted into the pAS2–1 and pACT2 vectors and tested for pairwise interactions using the yeast two hybrid system. For these experiments, the yeast co-transformants were grown and the lysates tested for β-galactosidase activity. The results were compared on the basis of relative specific activities (arbitrary units of β-galactosidase activity per unit of cell culture density). The results are shown in Fig. 6. This analysis confirms the biochemical data that p125 and PCNA interact and that p50 and PCNA do not interact. F6

The N2 Peptide Binds to PCNA—Previous work had identi-

[2] J.-Y. Mo and M. Y. W. T. Lee, manuscript in preparation.

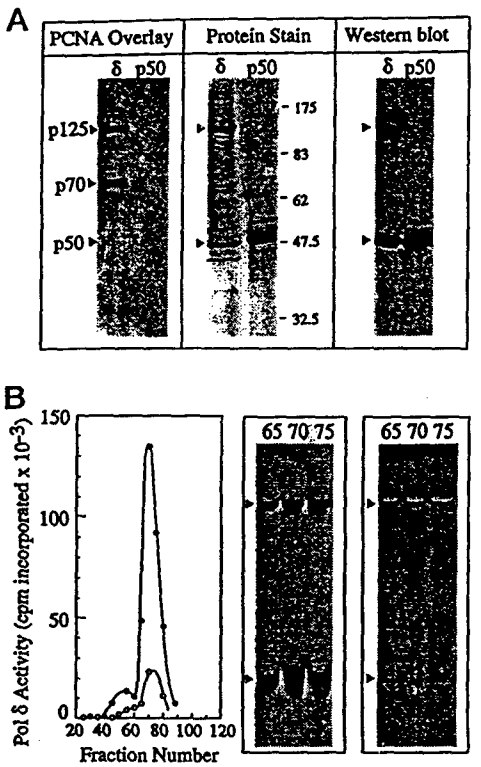


FIG. 4. **PCNA overlay analysis of Pol δ.** PCNA was labeled with biotin by reaction with biotinamidocaproate *N*-hydroxysuccinamide ester (see under "Experimental Procedures"). Samples were run on SDS-PAGE gels (10% acrylamide), transferred to nitrocellulose, and then blotted using biotinylated PCNA/streptavidin-horseradish peroxidase conjugate. The blots were visualized using a chemiluminescence method (ECL detection system, Amersham Pharmacia Biotech). *A*, the *left panel* shows an experiment using purified calf thymus pol δ obtained by immunoaffinity affinity chromatography (21) that had been further purified on heparin-agarose. This enzyme (δ) was analyzed by PCNA overlay together with recombinant p50 (*p50*) overexpressed in *E. coli* (16) and purified to near-homogeneity (2.5 μg of protein were used). The positions of the p125 and p50 subunits of pol δ are shown by the *arrowheads*. Also shown is the position of a p70 polypeptide that interacts with PCNA. The *center panel* shows the Coomassie Blue-stained SDS-PAGE gel of the same preparations, and the *right panel* is a Western blot using a mixture of monoclonal antibodies to p125 and p50 to show the identity of the polypeptide bands. *B*, p125 and p50 were overexpressed in Sf9 cells and purified to near homogeneity (P. Zhang and M. Y. W. T. Lee, unpublished data). The *left panel* shows the elution of pol δ activity from the final Mono-Q chromatography step, assayed in the absence (○) and presence (●) of PCNA. The *center panel* shows a Western blot of the peak fractions 65, 70, and 75 with a mixture of antibodies to the p125 and p50 subunits. The *right panel* shows the overlay blot with biotinylated PCNA. The *arrowheads* show the migration positions of p125 and p50 subunits.

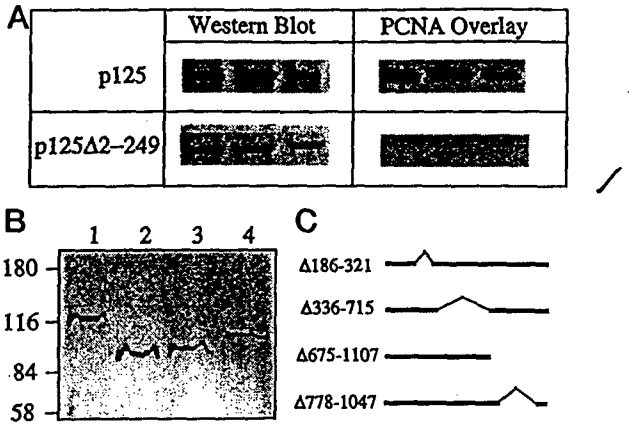


FIG. 5. **PCNA overlay of recombinant p125 and its N-terminal deletion mutants.** *A*, p125 and its N-terminal deletion mutant (Δ2–249) in which residues 2–249 are deleted were expressed in Sf9 cells and analyzed by PCNA overlay. The three *lanes* are the three peak fractions of the recombinant proteins during high pressure liquid chromatography gel filtration that were subjected to SDS-PAGE and overlay analysis with biotinylated PCNA. The *left panels* show the Western blots of the fractions for p125 and the 97 kDa Δ2–249 deletion mutant. The *right panels* show the overlay with biotinylated PCNA. *B*, Sf9 cells (1×10^6) were infected with recombinant baculovirus vectors for Δ186–321, Δ336–715, Δ675–1107, or Δ778–1047. Cell lysates (50 μl) from each of the cultures were then subjected to SDS-PAGE and analyzed by overlay with PCNA as for *A*. *Lanes 1–4*, represent Δ186–321, Δ336–715, Δ675–1107, and Δ778–1047, respectively. *C*, map of the deletion mutants Δ186–321, Δ336–715, Δ675–1107, and Δ778–1047.

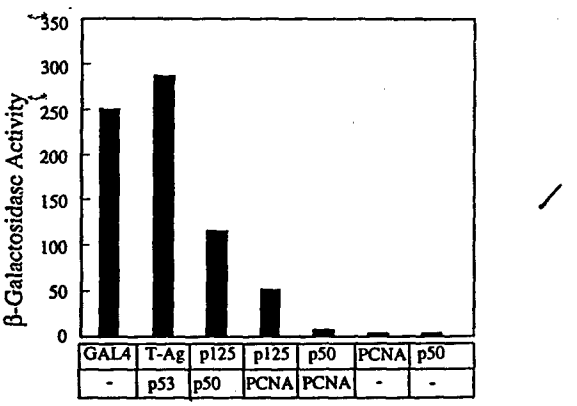


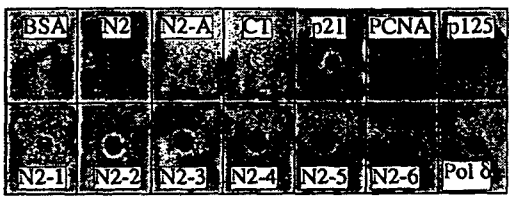
FIG. 6. **Liquid assay for yeast two hybrid interactions between p125 and PCNA.** The p125, p50, and PCNA sequences were tested for pairwise interactions in the yeast two hybrid system as described under "Experimental Procedures." The data are shown as arbitrary units of β-galactosidase activity per unit of cell culture density. T-antigen and p53 constructs were used as controls for a known pair of interacting proteins. The following pairs were tested: p125-p50, p125-PCNA, and p50-PCNA.

fied a region of the N terminus of pol δ, the N2 region (GVTDEGFSVCCHIHGFAPYFY, residues 129–149) as being involved in the interaction of pol δ with PCNA. This was based on the ability of a synthetic peptide with this sequence to inhibit the PCNA stimulation of pol δ (10). However, these experiments were performed with purified pol δ, and in the context of evidence that p125 does not interact with PCNA in other systems, it could be argued that these findings were due to an interference of the peptide with an interaction between p125 and an intermediary protein that leads to loss of PCNA response. Experiments were performed to test for a direct interaction of PCNA with the N2 peptide as well as with several variants with selected alanine replacements by dot blot analysis using biotinylated PCNA (Fig. 7). The peptides were bound to nitrocellulose and then blotted with biotinylated PCNA. The results (Fig. 7) show that immobilized N2 peptides, but not the mutant N2 peptide in which the three terminal YFY residues were replaced with alanine (GVTDEGFSVCCHIHGFAP**AAA**), are blotted by biotinylated PCNA. p21 peptide and p125 were also blotted in this dot blot assay (Fig. 7). The loss of interaction of the mutant in which the three terminal aromatic residues were changed to alanine is highly significant because it provides supportive evidence that the N2 region contains a variant of the PCNA binding motif (see under "Discussion").

DISCUSSION

Biochemical evidence for a direct interaction of the p125 catalytic subunit with PCNA was obtained. The demonstration of a physical complex between recombinant p125 and PCNA by gel filtration and chemical cross-linking with sulfo-EGS provides rigorous evidence for this interaction. In addition, the use of biotinylated PCNA in overlay experiments also shows that

this interaction is not dependent on the presence of other pol δ subunits, and the use of deletion mutants of p125 restricts the location of the interaction site to the N-terminal 186 residues of p125. Further evidence for a region on p125 that is involved in the interaction with PCNA was obtained by the use of synthetic peptides to the N2 region (residues 129–149). These findings confirm and extend previous studies from this laboratory that show that p125 directly interacts with pol δ and that the N2 region in p125 can be implicated in the protein-protein interaction with PCNA. The need for a more detailed investigation of whether there is a direct interaction between p125 and PCNA was raised by studies of pol δ-PCNA interactions in yeast, in which only negative evidence for an interaction of the p125 and PCNA has been obtained. Tratner *et al.* (18) reported that recombinant *S. pombe* p125 did not interact with hemagglutinin epitope-tagged PCNA when they were co-expressed in insect cells and tested for co-immunoprecipitation of ^{35}S-labeled proteins or when tested for pairwise interactions by the yeast two hybrid system. In *S. cerevisiae*, a third subunit of pol δ encoded by the POL32 gene has been identified (22, 23). A ^{32}P-tagged PCNA containing a fused PKA site at the N terminus was used in overlay experiments against p125 (POL3), p58 (POL31), and p55 (POL32) subunits. The results showed that only the p55 subunit bound to the tagged PCNA and no evidence of interaction of either p58 (the p50 homologue) or p125 with PCNA was found (19). The different results that were obtained in the yeast system could be due to differences in the experimental conditions used, as noted by Eissenberg *et al.* (19). The present studies confirm that, unlike the yeast proteins, human PCNA and p125 co-immunoprecipitate with either p125 or PCNA antibodies when they are co-expressed in Sf9 cells, COS 7 cells, and are also co-immunoprecipitated from calf thymus or HeLa extracts. As already noted, co-immunoprecipitation methods do not eliminate the possibility that positive results are due to the intervention of an intermediary polypeptide(s).



N2 GVTDEGFSVCCHIHGFAPYFY
N2-A GVTDEGFSVCCHIHGFAPAAA
N2-1 GVTDEGASVCCHIHGFAPYFY
N2-2 GVTDEGFRVCCHIHGFAPYFY
N2-3 GVTDEGFSVACHIHGFAPYFY
N2-4 GVTDEGFSVCCAIHGFAPYFY
N2-5 GVTDEGFSVCCHIAGFAPYFY
N2-6 GVTDEGFSVAAHIHGFAPYFY
CT DLEDQEQLLRRFGPPGPEAW
p21 GRKRRQTSMTDFY
HSKRRLIFS

FIG. 7. Dot blot analysis of binding of PCNA using biotinylated PCNA. Synthetic peptides/proteins were dot blotted onto nitrocellulose membranes and tested for binding to PCNA using biotinylated PCNA as described under "Experimental Procedures." The proteins tested were bovine serum albumin (*BSA*), recombinant p125 subunit expressed in Sf9 cells (*p125*), and immunoaffinity purified calf thymus pol δ (*pol δ*). The synthetic peptides that were tested are listed below the blot. *CT* is a peptide to the C terminus of p125 (residues 1091–1107) that we have used for the preparation of monoclonal antibody 38B5. *p21* is the p21-derived peptide (139–160) that contains the consensus PCNA binding motif (*underlined*).

An important result obtained in these studies is the first biochemical demonstration of a complex between free recombinant p125 and PCNA by gel filtration. All the gel filtration studies were performed in the presence of 150 mM NaCl, a standard biochemical practice to avoid nonspecific associations. Thus the association between p125 and PCNA takes place at ionic strengths that are near physiological. The cross-linking of the complex with sulfo-EGS showed that there was a rapid cross-linking to a high molecular weight species of a size that was consistent with a p125-PCNA trimer. These results must be taken in the context that under the conditions used we have reproducibly found that PCNA itself is only slowly cross-linked to the trimer and that the bulk of the reaction products are the monomer and dimer species (5). Previous observations from this laboratory have shown that PCNA in solution is an equilibrium mixture of the dimer and trimer species (5). The findings that the only complex present was a PCNA trimer-p125 complex, with an absence of dimeric or monomeric PCNA complexes with p125, suggests that p125 either selectively binds to the PCNA trimeric form or stabilizes the PCNA trimer. This preference for trimeric PCNA is consistent with the physiologically expected interaction of pol δ with PCNA, the function of which is associated with a trimeric state. It is also consistent with current models of the assembly of the replication complex,

TABLE I
Alignment of the N2 region of Pol δ with the PCNA binding motif

			PCNA Binding Motif	Gen Bank no.
			12345678 901234567	
Pol δ	S. cerevisiae	151	CNV TGFKNYLY VPAPNSSDA	X61920
Pol δ	*S. pombe*	130	VHV VGFLPYFY VKAPVGFRP	LO7734
Pol δ	Human	139	CHI HGFAPYFY TPAPPGFGP	M81735
UDG	Human	1	MIG QKTLYSFF SPSPARKRH	X89398
MCMT	Human	158	STR QTTITSHF AKGPAKRKP	X63692
Tigger Cds2	Human	86	LMR QTSLLSYF KKLPQPPQP	U49973
Rad2	*S. pombe*	335	TIP QGRLDSFF KPVPSSPKK	X77071
RAD27	*S. cerevisiae*	337	SGI QGRLDGFF QVVPKTKEQ	P26793
DNA ligase I	Human	1	M QRSIMSFF HPKKEGKAK	M36067
p57	Human	266	KLS GPLISDFF ATRKRSAPE	U48869
$p21^{Waf1/Cip1}$	Human	141	KRR QTSMTDFY HSKRRLIFS	P38936
p21	*Drosophila*	181	RKR QPKITEFM KERKRAAQA	U68477
POL32	*S. cerevisiae*	335	LKK QGTLESFF KRKAK*	Z49543
UDG	*S. cerevisiae*	18	KRK QTTQEDFF GTKKSTNEA	J04470
Cdc27	*S. pombe*	359	KPQ QKSIMSFF GKK*	M74062
POGO	*Drosophila*	489	AVL QKKITDYF*	S20478
FEN1	Human	334	GST QGRLDDFF KVTGSLSSA	P39748
XPG	Human	987	QQT QLRIDSFF KLAQQEKED	P28715

UDG, uridine DNA glycosylase; MCMT, methyl 5' cytosine DNA methyl transferase. The eight residue PCNA binding motif is numbered 1–8 at the top. Conserved residues are double-underlined at positions 1, 4, 7, and 8. In the C-terminal regions (numbered 9–17), proline residues are single underlined and basic residues (arginine and lysine) are double underlined.

in which RFC first loads PCNA onto DNA, following which pol δ is recruited (8). Recent findings (24) have shown that RFC disengages from PCNA upon sliding clamp formation and that the loaded PCNA clamp was able to recruit and bind polymerase δ and stimulate DNA replication.

Recently, a number of additional proteins that bind to PCNA have been identified (4, 9, 11, 25). These findings have major implications for an understanding the roles of PCNA. These PCNA-binding proteins fall into three major groups: DNA replication proteins (pol δ, RFC and pol ϵ, FEN1, and DNA ligase), DNA repair proteins (XPG and methyl(5-cytosine)methyl transferase), and cell cycle regulatory proteins (p21 and p57). These proteins contain a short PCNA binding motif in which there is a conserved glutamine and two conserved aromatic residues. The motif in question lies at the N terminus of the p21 peptide that forms an α-helical region in which the two aromatic residues interact with the large hydrophobic pocket of PCNA, whereas the C-terminal region forms an anti-parallel β sheet with the interdomain connector loop of PCNA (7).

An obvious question that arises is whether the N2 region of the p125 subunit of pol δ harbors a sequence that corresponds to the PCNA binding motif that has been identified in these PCNA-binding proteins. The alignment of the N2 sequence of pol δ from human and yeast with the PCNA binding motifs of a number of proteins is shown in Table I, which shows the eight-residue motif bounded by the conserved glutamine and the aromatic residues. The main features of the motif are the conserved glutamine at position 1, the presence of an aliphatic residue (leucine or isoleucine) at position 4, and a pair of aromatic residues at positions 7 and 8. Comparison of the N2 sequence shows that it possesses the aromatic residues and the aliphatic residue at position 4, but does not have the conserved glutamine. Thus, this region of the N2 sequence does not carry a complete consensus with the known PCNA binding motif. However, this glutamine residue is not conserved in p57, which also binds PCNA (Table I). Evidence obtained in this study using peptides to the N2 region of pol δ provides strong evidence that it contains a variant of the PCNA binding motif (Table I). The loss of binding when the aromatic residues are mutated in the N2 peptide does provide significant evidence that this region of the N2 sequence may be a member of the PCNA binding motif family of sequences.

In Table I, the regions C-terminal to the PCNA binding motif are also aligned. In the case of p21, this region is involved in the second major protein-protein contact of the peptide with PCNA and forms an anti-parallel β-sheet with the interdomain connector loop of PCNA (7). In previous studies, it had been shown that the interdomain connector loop of PCNA is important for the interaction of pol δ, and it was proposed that the N2 region of pol δ may interact with PCNA in a manner that may be similar to the interaction of p21 with PCNA (26, 27). There is a group of PCNA-binding proteins in which there is a preponderance of basic residues in this region (Table I), as previously noted by Warbrick *et al.* (28). However, it is also seen that there is a second group of sequences, in which there is a high percentage of proline residues. This includes pol δ from human and yeasts, human uracil DNA glycosylase, methyl 5′-cytosine methyl transferase, the human Tigger sequences (28), and *S. pombe* RAD2. The positions of the prolines is well conserved in these eight examples (Table I). The presence of this "proline-rich" motif provides additional support for the view that the N2 sequence belongs to the family of PCNA binding sequences. Furthermore, it should not be forgotten that from the example of p21 that the Q*XXI*/L*XX*FF motif reflects an interaction only with the large hydrophobic pocket of PCNA. Although examples also exist that indicate that this interaction alone (*Drosophila* POGO and *S. pombe* Cdc27) is sufficient for PCNA binding, the presence of the two features of the C-terminal region in the form of the proline rich motif and the basic motif suggests that these may represent sequences that are suited to the formation of a β-sheet with the interdomain connector loop of PCNA.

In summary, a detailed approach was undertaken to investigate the issue of whether the p125 polypeptide directly interacts with PCNA. Our findings provide strong biochemical confirmation that there is a direct interaction between the p125 subunit and PCNA, although they do not eliminate the possibility that the pol δ holoenzyme has multiple sites of interaction with PCNA through one or more of its subunits. The latter possibility is one that could be facilitated by the trimeric nature of PCNA, because this provides for extended interactions of individual PCNA subunits with different subunits of pol δ.

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Identification of DNA replication and cell cycle proteins that interact with PCNA

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ABSTRACT

The identity of DNA replication proteins and cell cycle regulatory proteins which can be found in complexes involving PCNA were investigated by the use of PCNA immobilized on Sepharose 4B. A column containing bovine serum albumin (BSA) bound to Sepharose was used as a control. Fetal calf thymus extracts were chromatographed on PCNA–Sepharose and BSA–Sepharose. The columns were washed and then eluted with 0.5 M KCl. The salt eluates were examined for the presence of both DNA replication proteins (Pol α, δ, ε, PCNA, RFC, RFA, DNA ligase I, NDH II, Topo I and Topo II) and cell cycle proteins (Cyclins A, B1, D1, D2, D3, E, CDK2, CDK4, CDK5 and p21) by western blotting with specific antibodies. The DNA replication proteins which bound to PCNA–Sepharose included DNA polymerase δ and ε, PCNA, the 37 and 40 kDa subunits of RFC, the 70 kDa subunit of RPA, NDH II and topoisomerase I. No evidence for the binding of DNA polymerase α, DNA ligase I or topoisomerase II was obtained. Of the cell cycle proteins investigated, CDK2, CDK4 and CDK5 were bound. This study presents strong evidence that PCNA is a component of protein complexes containing DNA replication, repair and cell cycle regulatory proteins.

INTRODUCTION

The discovery of a stimulating factor for DNA polymerase δ (1,2) that eventually led to its identification as proliferating cell nuclear antigen (PCNA) (3) stimulated major advances in our understanding of DNA synthesis at the replication fork. PCNA functions as a sliding clamp which endows pol δ with a high degree of processivity (4). Studies of *in vitro* SV40 DNA replication have now led to a fuller understanding of the protein machinery required for the formation of a functional mammalian DNA replication fork in which DNA polymerase δ (pol δ) and PCNA play a central role. The current model is one in which replication factor C (RFC, also known as activator-1), a complex of five subunits, first binds to the primer-template terminus and loads the PCNA onto the 3′ hydroxyl end of the primer strand of the DNA primer-template in an ATP-dependent process. Following the formation of a RFC/PCNA complex, pol δ is then recruited to assemble an elongation complex that catalyzes DNA synthesis in the presence of deoxynucleotide triphosphates (5–9). Replication protein A (RPA), a ssDNA binding protein, is involved in both initiation and elongation, as it stimulates pol δ activity in the presence of RFC and PCNA (6,10). A DNA helicase activity is essential to the replication machinery and serves mainly to unwind replication origins during the initiation phase of DNA replication and to separate parental DNA strands during the elongation phase. A helicase which is highly associated with pol δ has been isolated (11). Recently, six human helicases have been purified to near homogeneity (12). Furthermore, a nuclear DNA helicase II (NDH II) has also been purified (13). Like the large T antigen of SV40, it was found to unwind both DNA and RNA. Molecular cloning of NDH II revealed a high homology to human RNA helicase A (14). Pol α/primase is primarily involved in the synthesis of RNA primers plus short stretches of DNA primers on the lagging strand, while the actual elongation of the primers is performed by pol δ in a process requiring polymerase 'switching' (15). A topoisomerase activity is also required, and studies using the SV40 system showed that either topoisomerase I or topoisomerase II is capable of removing positive supercoils ahead of the replication fork (16,17). The model of the protein assembly at the replication fork now resembles that of the well defined prokaryotic systems, requiring the presence of two pol δ molecules (18).

A major area of research which is currently emerging is the exploration of the biochemical and genetic mechanisms by which cell cycle regulation of DNA synthesis is achieved. There have been rapid advances in delineating the existence of cell cycle proteins: these include the cyclins A and B, a family of G1 cyclins (E, D cyclins) and a family of cyclin dependent kinases (CDKs) (19,20). There is now evidence for the cell cycle control of mammalian DNA replication by the cyclin–CDK system (21). A number of studies point to the existence of protein–protein interactions of DNA synthesis proteins with cell cycle dependent protein kinases or cyclins, as well as the phosphorylation of DNA synthesis proteins by CDKs. DNA polymerase α is phosphorylated in a cell cycle specific manner and is a substrate for $p34^{cdc2}$ (22,23). The RPA complex purified from HeLa cells or Manca cells is also phosphorylated in a cell cycle dependent manner by one or more members of cyclin/CDK2 family, and its phosphorylation has been shown to stimulate the initiation of SV40 DNA synthesis *in vitro* (24,25). Recent studies by Pan *et al.* (26) showed that both CDK2/cyclin A and DNA-dependent protein

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kinase phosphorylate the 34 kDa subunit of RPA. However, phosphorylated and unphosphorylated forms of RPA were equally active in SV40 DNA replication and nucleotide excision repair (26). Using immunoprecipitation and western blot experiments, Xiong *et al.* (27,28) showed combinatorial interactions of D type cyclins, cyclin-dependent kinases with PCNA and with p21. p21, also known as WAF1, CIP1 or Sdi1, is an inhibitor of the CDKs that control the initiation of the S phase of the cell cycle and DNA replication. The N-terminal region of p21 contains the CDK inhibitory domain whereas the C-terminal region contains a PCNA binding domain that leads to the inhibition of DNA synthesis (29).

In addition, both pol δ and PCNA have been shown to be required for DNA repair (30). Thus, PCNA, through its interactions with elements of both the DNA replication apparatus and the cell cycle regulatory system, has emerged as an important locus for protein–protein interactions that may provide communication between DNA replication, DNA repair and cell cycle control. Definition of the number and nature of these protein–protein interactions will therefore be important. In this study, immobilized recombinant PCNA is used as a means for the isolation of proteins that bind to PCNA.

MATERIALS AND METHODS

Immunoblotting

After electrophoresis in 5–15% gradient gels, proteins were transferred to nitrocellulose membranes. Prestained protein standards (Sigma Chemical Co.) were used as molecular weight markers and provided visual confirmation of efficient transfer. The nitrocellulose blots were incubated with 3% BSA in phosphate-buffered saline as a blocking agent. The blot was then incubated with the primary monoclonal antibody at a final concentration of 5 μg/ml or with a polyclonal antibody at ~1:500 dilution for 12 h at 25°C. After washing, the blot was incubated with biotinylated sheep anti-mouse immunoglobulin, followed by streptavidin-biotinylated peroxidase preformed complex. When polyclonal antibodies were used, the second antibody was anti-rabbit IgG biotinylated species-specific whole antibody instead of anti-mouse IgG. Color development was performed by incubation with 4-chloro-1-napthol and hydrogen peroxide and was terminated with sodium azide.

Antibodies used were as follows: polyclonal antibodies against the p145, p40, p37 and p38 subunits of RFC (Dr J.Hurwitz, Memorial Sloan Kettering Cancer Center, NY); polyclonal antibodies against the p70 and p11 subunits of RPA and monoclonal antibody against the p34 subunit of RPA (Dr S.H.Lee, St. Jude's Children's Hospital, Memphis, TN); polyclonal antibodies to human topoisomerase I and II (ToPoGen Inc.); PCNA monoclonal antibody mAB19F4 (American Biotech. Inc., Plantation, FL); p21 monoclonal antibody (Santa Cruz); monoclonal antibody against DNA polymerase ε (Dr J.E.Syvaoja, University of Oulu, Finland); monoclonal antibody against polymerase α (American Type Culture Collection); DNA ligase I polyclonal antibody (Dr A.Tomkinson, University of Texas Health Science Center at San Antonio); rabbit antiserum to mouse cyclins D1, D2 and D3 (Dr C.J.Sherr, St. Jude's Children's Hospital, Memphis, TN); monoclonal antibodies to cyclins A, B1 and E and to both CDK2 and CDK5 (Dr E.Lee, Massachusetts General Hospital, Boston); polyclonal antibody to CDK4 (Dr S.Hanks, Vanderbilt University, TN); monoclonal antibody to NDH II (Dr F.Grosse, Heinrich-Pette Institute for Experimental Virology and Immunology, Germany).

Preparation of PCNA and BSA affinity columns

Recombinant PCNA was overexpressed in *E.coli* and purified to homogeneity as previously described (31). Activated CH–Sepharose, which allows coupling to a six carbon spacer arm, was obtained from Pharmacia LKB Biotech. Purified recombinant PCNA (20 mg in 50 ml) was dialyzed against 2 l of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0 (four changes at 6–8 h intervals). Activated CH–Sepharose 4B (2 g) was suspended in 30 ml of cold 1 mM HCl. The gel was washed in a column with 400 ml of cold 1 mM HCl followed by 200 ml of 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0. The coupling reaction was performed by addition of PCNA (20 mg, 50 ml in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.0) to the washed gel. The suspension was rotated end over end for 18 h at 4°C. The protein content of the supernatant was checked at intervals by absorbance at 280 nm to monitor the progress of the reaction. After an overnight reaction it was estimated that ~2 mg PCNA was bound/ml of gel. The suspension was centrifuged and the supernatant discarded. The gel was then suspended in 50 ml of 1 M ethanolamine, pH 9.0 for 18 h to block unreacted groups. The gel was washed with 200 ml of 1 M NaCl–0.1 M sodium acetate, pH 6.0 followed by 200 ml of 1 M NaCl–0.1 M Tris–HCl, pH 8.0 and 200 ml of 0.5 M NaCl–0.1 M Tris–HCl, pH 8.0. The gel was equilibrated with TGEED buffer (50 mM Tris–HCl, pH 7.8, 10% glycerol, 0.5 M EDTA, 0.1 mM EGTA and 1 mM dithiothreitol). A control column in which bovine serum albumin (BSA) was substituted for PCNA was prepared by the same procedure. In this case ~8.4 mg of BSA were coupled to 5 ml of activated CH–Sepharose 4B. All operations were performed at 4°C.

Preparation of calf thymus extracts

Frozen fetal calf thymus tissue (10 g) was used to prepare 50 ml of tissue extract. The tissue was homogenized in a blender with 50 ml of lysis buffer (50 mM Tris–HCl, pH 7.8, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM $MgCl_2$, 0.25 M sucrose, 10% glycerol, 10 mM KCl, 0.1 mg/ml soybean trypsin inhibitor, 1 mM benzamidine, 0.1 mg/ml bacitracin, 1 mg/ml pepstatin A, 1 mg/ml leupeptin, 0.2 mM phenylmethyl sulfonyl fluoride and 10 mM sodium bisulfite). The homogenate was centrifuged for 1 h at 15 000 *g*. The extract was then centrifuged at 100 000 *g* for 1 h. All operations were performed at 4°C.

Affinity chromatography

Affinity chromatography was performed by mixing 50 ml of calf thymus extract with the PCNA–Sepharose (5 ml) and rotating the suspension end over end for 2 h. The gel was then packed into a column and washed with 100 ml of 50 mM KCl in TGEED buffer and eluted with 0.5 M KCl in TGEED buffer. Fractions of 0.3 ml were collected. Control experiments in which immobilized BSA was used as the column support was performed in parallel.

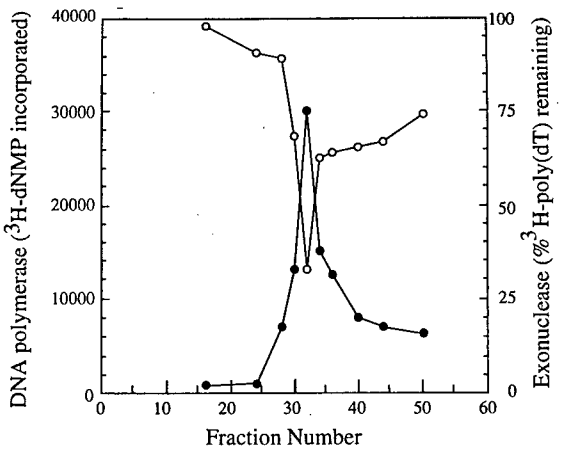

Figure 1. Affinity chromatography of calf thymus extract on PCNA–Sepharose. Crude calf thymus extract (50 ml) was rotated end over end with 5 ml of PCNA–Sepharose for 2 h. The gel was then packed onto a column, washed with 100 ml of 50 mM KCl TGEED buffer and stripped with 0.5 M KCl in TGEED (Materials and Methods). Fractions (0.3 ml) were collected and assayed for polymerase activity using poly dA/oligo dT as a template in the presence of PCNA (closed circles) and for exonuclease activity using [^{3}H]dT$_{50}$ (open circles) as previously described (1).

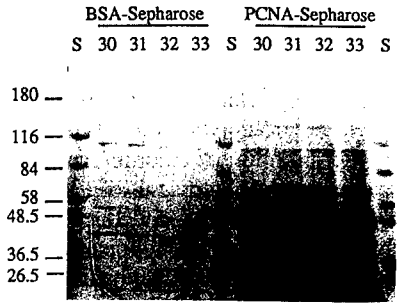

Figure 2. SDS–gel electrophoresis of proteins bound to PCNA and BSA affinity columns. Fractions 30–33 from the BSA-control column and the PCNA column were subjected to SDS–PAGE and stained for protein. From left to right are fractions 30–33 from the BSA control column, followed by fractions 30–33 from the PCNA column. The latter fractions correspond to the peak of pol δ activity. S: pre-stained protein standards (Sigma Chem. Co., α-2-macroglobulin, 180 kDa; β-galactosidase, 116 kDa; fructose 6-phosphate kinase, 84 kDa; pyruvate kinase, 58 kDa; fumarase, 48 kDa; lactate dehydrogenase, 36 kDa; triosephosphate isomerase, 26 kDa).

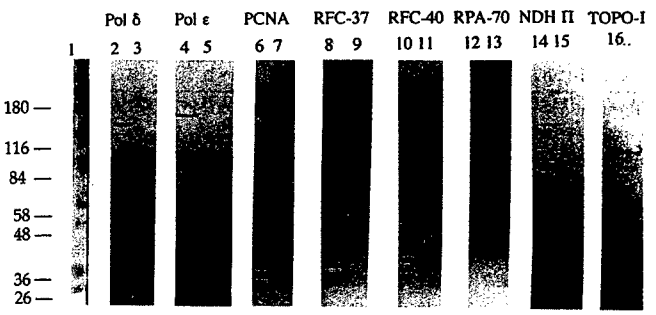

Figure 3. Immunoblots against DNA replication proteins bound to the PCNA affinity column. Lane 1: molecular weight markers with weights in kDa as indicated. Lanes 2, 4, 6, 8, 10, 12 ,14 and 16 each illustrate fraction 32 from the PCNA column western blotted against pol δ, pol ε, PCNA, RFC-37, RFC-40, RPA-70, NDH II and topoisomerase I antibodies, respectively. Lanes 3, 5, 7, 9, 11, 13 and 15 depict fraction 32 eluted from the BSA column and blotted against the same antibodies. Immunoblots were performed as described in Materials and Methods.

RESULTS

Affinity purification of DNA replication complex on PCNA–Sepharose

Calf thymus extracts were chromatographed on PCNA–Sepharose as described in Materials and Methods. The eluted fractions were assayed for DNA polymerase δ activity using poly dA/oligo dT as a template and for exonuclease activity using [^{3}H]dT$_{50}$. Preliminary experiments established that pol δ was bound to the column and was eluted at ~250 mM KCl when a KCl gradient was applied (not shown). A standard protocol was then used in which the bound material was eluted with 0.5 M KCl (Materials and Methods). No activity was detected in the flow through fractions, and both DNA polymerase and 3′→5′ exonuclease activities eluted together and were only detected in the eluate from the PCNA column (Fig. 1). (No activity was bound to a control BSA–Sepharose column when tested with a calf thymus extract.) The SDS–PAGE profile of polypeptides bound to PCNA–Sepharose and eluted with 0.5 M KCl is shown in Figure 2. A number of protein bands ranging from 18 to 210 kDa were present in the eluate from the PCNA–Sepharose column. The gels shown in Figure 2 were deliberately overloaded to show the presence of all bound polypeptides. Comparison with calf thymus extracts chromatographed on a control BSA column showed that there were several bands with two prominent polypeptides (110 and 43 kDa) that also adhered to the BSA column. Experiments were also performed in the presence of 1 mM ATP, since the interaction of PCNA with RFC is ATP dependent (6,7). However, the compositions of the polypeptides that were eluted were the same in the presence or absence of ATP (not shown).

The 0.5 M KCl eluate from the PCNA column was systematically tested for the presence of other replication proteins by western blotting. Representative blots are shown in Figure 3 from a number of individual experiments. The catalytic polypeptides of pol δ and ε were found to be present by western blot analysis using specific antibodies to pol δ (Fig. 3, lane 2) and pol ε (Fig. 3, lane 4). The pol δ antibody immunoblotted a band of 125 kDa, and the pol ε antibody detected a band of 145 kDa, in agreement with the previously reported molecular mass of pol ε isolated from calf thymus extracts (32,33). DNA polymerase α was not detected in the eluates by western blotting. PCNA itself was detected in the eluate as a 31 kDa band (Fig. 3, lane 6). This could be attributed either to stripping from the column, given that PCNA is trimeric, or due to an interaction of calf thymus PCNA subunits with immobilized PCNA. Antibodies against the individual 145, 40, 37 and 38 kDa subunits of RFC revealed positive results only for RFC-37 and RFC-40 (Fig. 3, lanes 8 and 10). Western blotting was also performed using antibodies to the 70, 34 and 11 kDa subunits of RPA. A positive blot was obtained for the 70 kDa subunit (Fig. 3, lane 12). These results indicate that both RFC and RPA are bound to the PCNA column.

The nuclear DNA helicase II (NDH II) enzyme was readily detected in the 0.5 M KCl eluate by immunoblotting as a 130 kDa band and three other bands of lower molecular weight ranging from 100 to 84 kDa (Fig. 3, lane 14). The lower molecular weight bands are likely to be proteolytic products. It has been reported that limited tryptic digestion of recombinant NDH II produced active helicases with molecular masses of 130 and 100 kDa (14). The presence of topoisomerase I and II, and ligase I was also tested for by immunoblotting. Only topoisomerase I was detected in the 0.5 M KCl eluate as a 100 kDa band (Table 1 and Fig. 3, lane 16). Examination of the fractions eluted from the BSA column by western blot yielded negative results for all of the above.

It is shown in this report that, in addition to pol δ, pol ε, RFC, RPA, PCNA, nuclear DNA helicase II (NDH II) and topoisomerase I are also present in the 0.5 M salt eluate from the PCNA column (Table 1). This collective elution of various constituents of the DNA replication machinery provides direct evidence for strong interactions between these proteins that directly or indirectly involve PCNA.

Table 1. Proteins which bind to immobilized PCNA as determined by western blotting of column eluates

Protein	PCNA column	BSA column	Protein	PCNA column	BSA column
Pol α	–	–	CDK2	+	–
Pol δ	+	–	CDK4	+	–
Pol ε	+	–	CDK5	+	–
PCNA	+	–	Cyclin A	–	–
RFC-37	+	–	Cyclin B1	–	–
RFC-38	–	–	Cyclin D1	–	–
RFC-40	+	–	Cyclin D2	–	–
RFC-145	–	–	Cyclin D3	–	–
RPA-11	–	–	Cyclin E	–	–
RPA-34	–	–	p21	–	–
RPA-70	+	–			
Ligase I	–	–			
NDH II	+	–			
Topo I	+	–			
Topo II	–	–			

Presence or absence in column eluates as determined by western blotting is shown as + or – respectively.

Binding of cell cycle regulatory proteins to PCNA–Sepharose

The PCNA–Sepharose column fractions containing peak pol δ activity were also tested for the presence of proteins involved in cell cycle regulation. Some principal components were detected through a series of western blots. Positive blots were obtained for cyclin dependent kinase 2 (CDK2), CDK4 and CDK5 (Fig. 4). Neither cyclins nor p21 were detected in the eluates (Table 1).

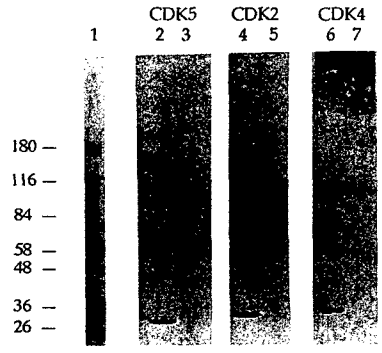

Figure 4. Immunoblots of cell cycle regulatory proteins eluting from the PCNA column. Lane 1: prestained protein markers with weights in kDa as shown. Lanes 2, 4 and 6: cell cycle regulatory proteins, CDK5 (31 kDa), CDK2 (33 kDa) and CDK4 (34 kDa), were bound by and eluted from the PCNA column. Shown also are the corresponding western blots of fraction 32 (lanes 3, 5 and 7) from the control BSA column where no CDK proteins were detected.

DISCUSSION

Recombinant PCNA was immobilized on Sepharose 4B and was systematically used to investigate the binding of replication and cell cycle proteins from fetal calf thymus extracts by affinity chromatography. As expected, tests for the binding of pol δ both by activity and by western blotting confirmed that it was bound. In addition, examination of the protein bands present in the peak of the bound fractions showed the presence of multiple polypeptide components. Some of these may represent adventitious binding although it may be noted that only a few bands with two prominent polypeptides of 110 and 43 kDa were observed in the eluates from the same fractions in the BSA control column. In addition to pol δ, several other replication proteins were bound to the affinity column. Specifically, the binding of PCNA, pol ε, RFC, RPA, NDH II and topoisomerase I was detected. The binding of pol δ and RFC was anticipated, since it is known that these proteins interact with PCNA (6). The collective elution of PCNA, pol δ, RFC and RPA was striking, in view of the fact that these are all components of the proposed replication complex involved in leading and lagging strand DNA synthesis (16,34).

The interaction of PCNA with pol ε is still controversial. Lee *et al.* (7) reported that RPA, RFC and PCNA could overcome the salt inhibition of DNA polymerase ε. Chui and Linn (35) observed strong inhibition of DNA polymerase ε by salt and found that this inhibition could not be completely overcome by RFC, RPA and PCNA which had little, if any, effect on the processivity of DNA polymerase ε. A major significance of these present findings is that they reveal a definite interaction, either direct or indirect, between pol ε and PCNA, thus linking pol ε to the replication fork. Navas *et al.* (36) have identified the DUN2 gene of *Saccharomyces cerevisiae* as DNA polymerase ε (Pol2). Mutations in the DUN2 gene displayed properties that suggest that pol ε has a role as a sensor of replication blocks and some forms of DNA damage, thus linking the DNA replication machinery to the S phase checkpoint (36). However, in *Schizosaccharomyces pombe* it was demonstrated that *cdc* 20^+ encodes the catalytic subunit of pol ε and the gene product is required for chromosomal replication but not for the S phase checkpoint (G.D'Urso, personal communication). A pol ε holoenzym

consisting of pol ε, PCNA, RPA and RFC may function on the lagging strand of the replication fork (37). This could provide a mechanism for proofreading in the lagging strand because pol ε, unlike pol α and similar to pol δ, has a 3′→5′ exonuclease activity (38,39). Zlotkin *et al.* (40), using UV crosslinking of nascent cellular DNA and immunoprecipitation, showed that DNA polymerase ε is essential in cellular nuclear DNA replication. Studies of *S.pombe cdc* 20^+ mutants showed that pol ε plays an important role in the elongation of nascent DNA chains, suggesting that pol ε participates in the switch from primer extension by pol α primase to leading strand synthesis (G.D'Urso, personal communication).

The presence of topoisomerase I in the eluates from PCNA–Sepharose is interesting, as it functions to relieve positive superhelicity during replication (41). The positive immunoblots for nuclear DNA helicase II (NDH II) (Fig. 3) in the peak eluates reveals the possibility of a complex involving the helicase enzyme. This is interesting in view of the fact that there may be differences between viral and host chromosomal DNA replication, so that there may be limitations of the *in vitro* SV40 replication system as a model system. Recently, a human nuclear protein that interacts with the constitutive transport element (CTE) of simian retrovirus was identified as RNA helicase A (42). The latter has a high degree of similarity to NDH II which also has RNA helicase activity (14). RNA helicase A was found to be concentrated in the nucleus in normal cells (42). It was also identified as an inherent shuttling protein that interacts with CTE *in vitro* and associates with CTE in its trafficking from the nucleus to the cytoplasm *in vivo* (42). Whether the presence of NDH II is physiologically relevant in the DNA replication complex purified from the PCNA–Sepharose affinity column is still an open question.

These findings are in concert with, and support other studies which have led to the partial purification of macromolecular complexes using conventional protein purification methods (43–45). Immobilized T4 bacteriophage gene 32 protein has been successfully used to characterize and isolate the interacting components of the T4 replication complex (46). The existence of a physical assembly of a mammalian replication complex, the 'replisome' has been inferred from studies of the prokaryotic system, and by consideration of the requirement for processive and uninterrupted DNA synthesis during replication. Evidence for the existence of such complexes is still fragmentary, and is based on the isolation of partially purified protein fractions by conventional methods that contain a number of replication proteins that can functionally replicate viral DNA (18,34). The advantage of an affinity chromatography approach over conventional methods is that it is rapid and is based on protein–protein interactions. This greatly lessens concerns that these complexes may be artifacts of the isolation methods. The current studies using affinity chromatography demonstrates the existence of a system of protein–protein interactions involving the replication proteins that could provide the molecular basis for the formation of a replication complex.

Since PCNA has been reported to bind to the cyclins (27,28), the binding of the cyclins and associated cyclin dependent kinases to the PCNA–Sepharose was also tested. Blots for the cyclin dependent kinases were positive in the case of CDK2, CDK4 and CDK5, while tests for associated cyclins were negative. This is surprising, since both CDKs and cyclins have been reported to associate in quaternary complexes with PCNA and p21 (27,28). It may be that tissue levels of the cell cycle proteins in calf thymus were too low for detection (28); also, expression of p21 occurs as a result of DNA damage (47). In general, a failure to observe any given protein in our experiments does not preclude its involvement in a replication complex, since the experiments are dependent on the sensitivity of the antibodies, the strength of the association and the stability of the given protein–protein interaction during purification. Recent studies have described several intermolecular interactions between cell cycle proteins and the replication proteins that may be of mechanistic significance in the cell cycle regulation of DNA replication. These include the demonstration that cyclin A or cyclin E–CDK complexes can trigger initiation of DNA synthesis (48), and that cyclin A is required for *in vitro* DNA replication (49). The phosphorylation of replication proteins by cyclin/CDKs has been demonstrated in the case of HSSB-p34 (50). In the latter case, cyclinA/CDK2 but not cyclinE/cdk2 was shown to phosphorylate HSSB. This critical observation indicates that targeting of the CDK2 to HSSB is necessary for phosphorylation to occur. Pol δ was reported to be phosphorylated *in vivo* (51). Recently, pol δ was found to be phosphorylated by cyclin/CDKs (Zeng and Lee, unpublished observations).

These studies which show the binding of both replication and cell cycle proteins to PCNA provides additional support for a central role of PCNA in the linkage of the processes of DNA replication and cell cycle regulation via protein–protein interactions. In addition, these findings demonstrate the existence of protein–protein interactions between DNA replication proteins and cell cycle regulatory proteins. This interaction of the cyclin–CDK cell cycle regulatory proteins with polymerases and elements of the DNA replication system could be important in understanding the cell cycle control of DNA replication. The findings that PCNA exhibits interaction with multiple protein partners suggest that it may have an important role in the formation of macromolecular complexes involved in DNA replication and its cell cycle control. For this reason, immobilized PCNA may be a particularly useful tool for the isolation of these complexes, a view that is supported by the studies reported. A molecular basis for the multiple protein partners with which PCNA interacts is now emerging, in the form of the recent identification of a short peptide consensus sequence which is found in several PCNA binding proteins including p21 (52), Fen1 (53) and cdc27 (54). There remain major questions as to the number and nature of the interacting protein partners of PCNA, and the mechanisms of how these interactions provide the necessary functional and regulatory outcomes in DNA replication and repair.

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